REMARKS

Claims 19 to 56 are pending in the application. Claims 19 to 24, 29, 30, 42, and 43 are currently under consideration.

Applicants acknowledge with appreciation the withdrawal of all rejections except for the rejection under U.S.C. § 112, first paragraph, for alleged lack of enablement.

Rejection under 35 U.S.C. § 112, first paragraph

The Examiner rejected claims 19 to 24, 29, 30, 42, and 43 under 35 U.S.C. § 112, first paragraph, as allegedly not being enabled. Action at page 2. Specifically, the Examiner alleged that "the specification, while being enabling for **a method for treating cancer comprising administering mAb74 or fragment thereof to induce apoptosis in Her2 overexpressing cells in cell culture (*in vitro*)**, does not reasonably provide enablement for **a method for treating cancer in a patient comprising administering an antibody or fragment thereof that binds HER2 and induces apoptosis in Her2 overexpressing cells *in vivo***." *Id.* (emphasis in original).

The Examiner conceded that the specification is enabling for cells in culture (*in vitro*), but repeatedly alleged that the specification does not enable methods of treating cancer in a patient (*in vivo*). Action at pages 3 to 4. Attempting to support this contention, the Examiner alleged that the art is unpredictable, stating:

> The unexpected nature of mAb74 indicates the antibody is novel and its properties regarding *in vivo* treatment of patients with cancer overexpressing HER2 are unknown and unpredictable because the specification states that the antibody's properties are unexpected, the specification does not provide guidance and examples for mAb74 or any antibody binding to HER2 to treat cancer by inducing apoptosis, and there is no relevant art teaching the treatment

> of cancer comprising administering an antibody that binds HER2 and induces apoptosis.

Action at page 5. The Examiner alleged later in the Office Action that

> Applicants do not provide guidance or examples for administering and discarding antibodies that do not effectively treat patients with HER2 overexpressing cancer. Applicants are enabled for monoclonal antibody mAb74 and antibodies that bind to the same epitope as mAb74, produced by hybridoma cell line ATCC No. 12078, which to bind HER2 and induce apoptosis in cells that are in cell culture, however, Applicants are not enabled for these antibodies to treat cancer in a patient because the specification does not enable the treatment of patients with HER2 overexpressing cancer comprising administering these antibodies with an unexpected, unpredictable property, and one of skill in the art would be forced into undue experimentation to practice the claimed invention for the reasons set forth above.

Action at page 14.

Applicants respectfully traverse. As the Examiner noted, the correct standard for determining enablement is whether it would have required "undue experimentation" to practice the claimed methods. *See* Action at page 4. Applicants assert that the question of enablement of the claimed methods can be separated into two inquiries. First, could one skilled in the art have made an antibody that binds Her2 and induces apoptosis in Her2 overexpressing cells using the guidance in the specification and the knowledge in the art without undue experimentation? Second, could one skilled in the art have used that antibody in a method for treating cancer characterized by overexpression of Her2 in a patient using the guidance in the specification and the knowledge in the art without undue experimentation?

Applicants assert that the answer to both questions is "yes." Furthermore, applicants assert that even if it is "surprising" or "unexpected" that an antibody that binds Her2 could induce apoptosis in Her2-overexpressing cells, any alleged

unpredictability with respect to that property is not relevant to the question of whether one skilled in the art could have used an antibody that has already been selected to have that property in a method for treating cancer. In other words, once the antibody with the property has been selected, it is not surprising or unexpected that it would retain that property in a method for treating cancer. In fact, one skilled in the art would expect the antibody to continue to have that property *in vivo*.

Turning now to the first question, applicants assert that the specification and the knowledge in the art at the time of filing enable one skilled in the art to make antibodies that bind Her2 and induce apoptosis in Her2 overexpressing cells without undue experimentation. For example, the specification teaches how to make an antibody or fragment thereof that binds to Her2 , *e.g*, at Example 2. The specification teaches how to select an antibody that induces apoptosis in Her2 overexpressing cells, *e.g.*, at Example 6.

Furthermore, in *Wands*, the Federal Circuit concluded that, in view of the appellant's specification, obtaining the monoclonal antibodies needed to practice the claimed invention would not require undue experimentation. *In re Wands*, 858 F.2d 731, 740 (Fed. Cir. 1988). The court stated that "[t]he nature of monoclonal antibody technology is that it involves screening hybridomas to determine which ones secrete antibody with desired characteristics." *Id.* at 740. Furthermore, "[t]he test is not merely quantitative, since a considerable amount of experimentation is permissible, if it is merely routine." *Id.* at 737. Thus, screening hybridomas to identify an antibody with the desired characteristics is routine, not undue, experimentation. Following *Wands*, one

must therefore conclude that it is not undue experimentation for one skilled in the art to make antibodies to Her2 and select those with desired characteristics, *i.e.*, antibodies that bind to Her2 and induce apoptosis in Her2 overexpressing cells. The antibodies recited in the present claims are therefore adequately enabled by the specification and the knowledge in the art.

Turning to the second question, applicants assert that the specification and the knowledge in the art at the time of filing enable one skilled in the art to use those antibodies in a method for treating cancer characterized by overexpression of Her2 in a patient. For example, the specification teaches how to make pharmaceutical compositions comprising an antibody or fragment thereof that binds Her2 and induces apoptosis in Her2 overexpressing cells, *e.g.*, at page 12, lines 3 to 14. The specification also teaches certain routes of administration for such pharmaceutical compositions, *e.g.*, at page 11, lines 29 to 33.

Attempting to support the contention that the claimed methods are not enabled, the Examiner alleged that "there is no relevant art teaching the treatment of cancer comprising administering an antibody that binds HER2 and induces apoptosis." Action at page 5.

Applicants respectfully traverse. Finding that the specification and the knowledge in the art at the time of filing adequately enables the claimed invention, does not require that the prior art disclose the exact method claimed. Rather, it is only required that one skilled in the art, using the teaching in the specification and the knowledge in the art at the time of filing, be able to practice the claimed method without

undue experimentation. Experimentation is permissible so long as it is not undue. Applicants assert that such experimentation may take the form of adapting known methods of administering agents, such as antibodies, to treat conditions in patients to the claimed method. Thus, the claimed methods may be enabled, for example, by the teachings of the specification, and the general knowledge in the art concerning administering antibodies to treat conditions in patients.

Applicants enclose copies of three review articles that demonstrate that certain methods of administering antibodies to treat conditions in patients were known in the art prior to the filing date of the present application. *See* Feldmann et al., "Cytokine expression and networks in rheumatoid arthritis: rationale for anti-TNFα antibody therapy and its mechanism of action," *J. Inflammation* 47: 90-96 (1996); Weiner et al., "Bispecific monoclonal antibody therapy of B-cell malignancy," *Leukemia and Lymphoma* 16:199-207 (1995); and Maini et al., "Monoclonal anti-TNF alpha antibody as a probe of pathogenesis and therapy of rheumatoid disease," *Immunol. Rev.* 144: 195-223 (1995). Each of those documents describes the successful treatment of a condition by administering an antibody to a patient prior to the filing date of the present application. Applicants assert that one skilled in the art could have applied the teachings of at least those documents, and the documents cited therein, and the guidance in the specification in order to carry out the claimed methods using the novel antibodies that bind to Her2 and induce apoptosis, without undue experimentation.

That conclusion is supported by Sasaki et al., "Monoclonal antibody induces apoptosis against cancer cells," *Nippon Rinsho* 60: 451-456 (2002), the English-

language abstract of which is enclosed (Sasaki). The authors note that an inhibitory humanized monoclonal antibody to ErbB-2 (Her2) "shows clinical response in some breast cancer patients, both with [the antibody] alone and in combination with Cisplatinum or other anti-cancer drugs." Sasaki. Furthermore, the authors discuss experiments involving a particular chimeric anti-ErbB-2 (Her2) antibody, CH401, that kills cancer cells by inducing apoptosis in Her-2 overexpressing cells both *in vitro* and *in vivo*. *Id.* Applicants assert that Sasaki supports the conclusion that even though the anti-Her2 antibodies recited in the present claims have an unexpected property, *i.e.*, induction of apoptosis in Her2-overexpressing cells, that property does not adversely affect the ability of one skilled in the art to use the antibodies in the claimed methods according to the teachings of the specification and the knowledge in the art.

Thus, applicants assert that first, the specification and the knowledge in the art at the time of filing would have enabled one skilled in the art to make an antibody that binds to Her2 and induces apoptosis in Her2 overexpressing cells, and second, the specification and the knowledge in the art at the time of filing would have enabled one skilled in the art to use that antibody in a method of treating cancer characterized by overexpression of Her2 in a patient.

Voskoglou-Nomikos

The Examiner alleged that "Voskoglou-Nomikos et al and the list of 60 human cancer cell lines used in the screen and maintained by the National Cancer Institute do not enable the extrapolation of in vitro results for the monoclonal antibody, MAB75, or any other HER2-binding antibody that induces apoptosis to cancer treatment or clinical

trial results." Action at page 8. Specifically, the Examiner alleged that Voskoglou-Nomikos "teach that the in vitro cell line model **might be** predictive in the case of typical **cytotoxic cancer agents but might fail to provide reliable information for at least some of the noncytotoxic cancer drugs**." *Id.* (Emphasis in original, citation omitted.)

Applicants traverse. First, applicants assert that an antibody that induces apoptosis in a cell is a cytotoxic agent. Apoptosis is also known as "programmed cell death," which means, as the name implies, that an agent that induces apoptosis causes the cell to die, *i.e.*, the agent is cytotoxic. Applicants therefore assert that the statement in Voskoglou-Nomikos concerning any alleged shortcomings of the National Cancer Institute Human Tumor Cell Line Screen (NCI Panel) with respect to noncytotoxic agents is not relevant to the present inquiry.

Second, applicants assert that the Examiner failed to address or refute the repeated assertions in Voskoglou-Nomikos that the NCI Panel not only is a good predictor of Phase II clinical trial results, but is a better predictor than mouse models or even human xenograft models. Specifically, Voskoglou-Nomikos states that

> **The *in vitro* cell line model was predictive for non-small cell lung cancer under the disease-oriented approach, for breast and ovarian cancers under the compound-oriented approach, and for all four tumor types together.** The mouse model was not predictive. The human xenograft model was not predictive for breast or colon cancers, but was predictive for non-small cell lung and ovarian cancers when panels of xenografts were used.

Voskoglou-Nomikos at Abstract (emphasis added). In addition, the authors state that

> When the mean $Log_{10}GI_{50}$ measure of preclinical activity was used, **the *in vitro* cell line model was found to be predictive of Phase II clinical performance** for NSCLC under the disease-oriented approach in breast and ovarian cancers under the compound-oriented approach and in the

> case of all four tumor types together. **Highly significant correlations were observed in all cases**, except colon cancer, when three consistent outlier data points corresponding to the mechanistically nontypical cytotoxic agents didemnin B, elsamitrucin, and rhizoxin were excluded in exploratory analysis.

Voskoglou-Nomikos at page 4235, paragraph bridging left and right columns. Finally, the authors state that

> **The work presented here argues for emphasis to be placed on *in vitro* cell lines** (in the context of the NCI Human Tumor Cell Line Screen) and appropriate panels of the human xenograft model.

Voskoglou-Nomikos at page 4237, left column, fourth full paragraph.

The standard for determining whether or not disclosed *in vitro* examples correspond to an *in vivo* activity is whether there is a reasonable correlation between the two. *See* MPEP § 2164.02 at 2100-195. Specifically, the MPEP states

> In other words, **if the art is such that a particular model is recognized as correlating to a specific condition, then it should be accepted as correlating** unless the examiner has evidence that the model does not correlate. Even with such evidence, **the examiner must weigh the evidence for and against correlation and decide whether one skilled in the art would accept the model as reasonably correlating to the condition**.

Id., citing *In re Brana*, 51 F.3d 1560, 1566 (Fed. Cir. 1995) (reversing the Board of Patent Appeals that *in vitro* data did not support *in vivo* applications) (emphasis added). Furthermore, according to the MPEP,

> **A rigorous or an invariable exact correlation is not required**, as stated in *Cross v. Iizuka*, 753 F.2d 1040, 1050, 224 USPQ 739, 747 (Fed. Cir. 1985):
>
> > [B]ased upon the relevant evidence *as a whole*, there is a reasonable correlation between the disclosed *in vitro* utility and an *in vivo* activity, and therefore **a rigorous correlation is not necessary where the disclosure of pharmacological activity is reasonable based upon the probative evidence**. (Citations omitted.)

MPEP § 2164.02 at 2100-196 (emphases added).

Applicants assert that Voskoglou-Nomikos supports the finding of a reasonable correlation between the *in vitro* activity exemplified in the specification and *in vivo* results. Applicants further assert that the Examiner has not presented any contrary evidence demonstrating that the NCI Panel is not a reasonable predictor of *in vivo* results.

The Examiner alleged that "the cell line MDA-MB-435 has a disputed origin and studies provide convincing data that the cells are of melanoma origin [rather than breast cancer origin]." Action at page 8. As noted by the Examiner, the apoptosis assays described in the specification were conducted using both MCF7 and MDA-MB-435 cells, both of which are part of the NCI Panel. *See* Example 6. The exemplary antibody that binds Her2 and induces apoptosis in Her2 overexpressing cells successfully induced apoptosis in both cell lines. Applicants assert that it is not relevant to the present claims, which recite a method for treating cancer, that MDA-MB-435 may be a melanoma cancer cell line rather than a breast cancer cell line. In either case, the exemplary antibody was able to induce apoptosis in at least two different cancer cell lines.

Drexler, Dermer, and Freshney

The Examiner alleged that Drexler teaches "a relevant example of 'acquisition or loss of certain properties during adaptation to culture systems.'" Action at page 9. The Examiner further alleged that Dermer and Freshney teach "the loss of phenotypic characteristics in cultured cells associated with their normal counterpart and 'petri dish

cancer' is a poor representation of malignancy, with characteristics profoundly different from the human disease. . . ." *Id.* The Examiner quoted the previous Office Action, alleging that

> "Clearly it is well known in the art that cells in culture exhibit characteristics different from those in vivo and cannot duplicate the complex conditions of the in vivo environment involved in host-tumor and cell-cell interactions", hence one could not extrapolate the in vitro results of the specification to the treatment of cancer.

Id.

Applicants traverse. First, applicants assert that the observations made in Drexler's study of Hodgkin and Reed-Sternberg cell lines is not applicable to the particular cell lines used in the present application. As discussed above, the present application uses cell lines from the NCI Panel to demonstrate the induction of apoptosis by an antibody or fragment thereof that binds to Her2. The cell lines of the NCI Panel have been identified as particularly valuable for the identification of anti-cancer therapeutic molecules. None of the cell lines discussed in Drexler is on the NCI Panel, nor does Drexler draw any conclusions about the NCI Panel from his observations concerning Hodgkin and Reed-Sternberg cell lines. In fact, Drexler notes that the development of Hodgkin and Reed-Sternberg cell lines has been particularly difficult compared to the development of other types of cancer cell lines. *See* Drexler at Abstract. Thus, for at least those reasons, Applicants assert that the conclusions in Drexler are inapplicable to the cell lines used to demonstrate the apoptotic activity in the present application.

Moreover, as discussed above, Voskoglou-Nomikos clearly demonstrated that the *in vitro* results obtained using cell lines on the NCI Panel reasonably correlate to the results of Phase II clinical trials. Applicants therefore assert that, contrary to the Examiner's contentions, one skilled in the art would conclude that the *in vitro* results described in the present application are reasonable predictors of *in vivo* results. Applicants assert that Drexler fails to refute that conclusion because Drexler addresses only two particular types of cancer cells that have already been found to be more difficult to develop into cell lines than other cancer types, and neither of those cell types was used in the present application.

The Examiner alleged that applicants did not address the Examiner's contentions with respect to Dermer and Freshney. Action at page 9. Applicants traverse. Applicants refer the Examiner to the discussion of those documents in the Response filed February 2, 2006, at pages 17 and 18. Applicants respectfully request that the Examiner consider those comments. Applicants will also discuss those documents briefly herein.

Dermer discusses the difficulties associated with using immortalized laboratory cell lines in certain types of cancer research. *See* Dermer. Dermer only discusses a single specific cell line, 3T3, which is not on the NCI Panel. Furthermore, Dermer urges that "models that mimic the human body and the developmental pathways of human cells, both normal and malignant, should be first identified. Only then will truly significant observations be made." Dermer at right column, third full paragraph. Applicants assert that the NCI Panel is just such a model. Voskiglou-Nomikos

demonstrates that the NCI Panel is predictive of *in vivo* Phase II clinical trial results. Thus, while the cell line discussed in Dermer, 3T3, may or may not be predictive of *in vivo* results, nowhere does Dermer address any of the cell lines used in the present application or on the NCI Panel. Nor does Dermer in any way directly address or refute the conclusions reached by Voskiglou-Nomikos concerning those cell lines.

In the Office Action mailed September 22, 2005, the Examiner cited page 4 of Freshey, alleging that it teaches "that it is recognized in the art that there are many differences between cultured cells and their counterparts *in vivo*." September 2005 Action at page 11. The Examiner further alleged that Freshney teaches that "[t]his has often led to tissue culture being regarded in a rather skeptical light." *Id.*

Applicants again note, however, that the very next sentence on page 4 of Freshney states that "[a]lthough the existence of such differences cannot be denied, **it must be emphasized that many specialized functions are expressed in culture and as long as the limits of the model are appreciated, it can become a very valuable tool**." Freshney at page 4, right column, second full paragraph (emphasis added). Furthermore, Freshney contains an entire chapter dedicated to "Cytotoxicity and Viability Assays." Freshney at Chapter 8, a copy of which is enclosed. In the Introduction to that chapter, the author discusses the use of *in vitro* cell lines for both pre-clinical evaluation of drugs and safety evaluation of chemicals, noting that

> [a]nimal models have always played an important role in both contexts, and although **cell culture systems have figured largely in the field of cancer chemotherapy, where the potential value of such systems for cytotoxicity and viability testing <u>is now widely accepted</u>**, there is increasing pressure for a more comprehensive adoption of in vitro testing in both spheres of application.

Freshney at page 183, first full paragraph (emphasis added). Thus, contrary to the Examiner's contention, Freshney supports applicants' assertion and Voskoglou-Nomikos' conclusion that one skilled in the art would find a reasonable correlation between *in vitro* results using certain cell lines and *in vivo* results.

Gura

The Examiner alleged that

> Gura teaches that the limitations of animal models has spurred the NCI to test drug candidates in cultures of human cells and now relies on 60 human tumor cell lines, however, over the last 7 years (as of 1997), the NCI-60 panel has been used to screen almost 63,000 compounds, of which 5,000 exhibited tumor cell killing activity. Computer screening to identify agents with novel mechanisms of action allow those selected agents to go to clinical trials. Gura teaches a method of screening anti-cancer drugs in human cell culture and identifying drugs that are of interest for clinical trials.

Action at page 10 (citations omitted). The Examiner further alleged that

> Gura teaches the unpredictability of anti-cancer drug activity and the massive screening required to identify potential drug candidates, which exemplifies the unpredictable activity of novel anti-cancer drugs, such as the novel antibody, mAb74.

Id. at page 10.

Applicants respectfully traverse. Applicants agree that Gura teaches that the limitations of animal models led the National Cancer Institute to develop and rely on a panel of 60 human tumor cell lines for identifying promising *in vivo* anti-cancer agents. Applicants disagree, however, with the Examiner's contention that Gura teaches "the unpredictability of anti-cancer drug activity and the massive screening required to identify potential drug candidates, which exemplifies the unpredictable activity of novel anti-cancer drugs, such as the novel antibody, mAb74."

Gura states that 63,000 compounds have been tested using the NCI Panel, of which 5000, or about 8%, exhibited tumor cell-killing activity. Gura at page 1042, left column, third full paragraph. First, applicants assert that the 63,000 compounds to which Gura refers were compounds with no known activity at the time they were screened using the NCI Panel. That is, applicants assert that Gura is describing the use of the NCI Panel for general high-throughput drug screening, in which a library of chemical compounds with no known activity is tested to identify those chemicals that may have anti-cancer activity. That situation has no bearing on the enablement of the present claims. Unlike Gura, applicants have already selected antibodies with at least one desired activity (i.e., the ability to bind Her2 on the surface of Her2 overexpressing cells), and have screened those antibodies for a second desired activity (i.e., the ability to induce apoptosis in Her2 overexpressing cells).

Furthermore, applicants assert that the enablement inquiry for the present claims can be divided into two inquiries: First, are the recited antibodies that bind to Her2 and induce apoptosis in Her2 overexpressing cells enabled? Second, is a method of treating cancer characterized by overexpression of Her2 in a patient using those antibodies enabled?

High-throughput screening of chemical compounds has little bearing on either question. With regard to the first question, *Wands* clearly supports a finding that such antibodies are enabled, because it involves only routine screening for one skilled in the art to make antibodies to a selected antigen (i.e., Her2), and then screen those antibodies for a desired property (i.e., induction of apoptosis in Her2-overexpressing

cells). With regard to the second question, whether the use of those antibodies in a method of treating cancer characterized by overexpression of Her2 in a patient is enabled, Gura has no comment. Gura contains no discussion whatsoever of whether the results of the NCI Panel screen are predictive of *in vivo* activity. Voskoglou-Nomikos *does* answer that question, however. Voskoglou-Nomikos found that *anti-cancer activity using the NCI Panel is predictive of* in vivo *activity*. Applicants assert that Gura does not alter the conclusion of Voskoglou-Nomikos, nor is Gura in any way contrary to a finding that the present claims are enabled.

Jain

The Examiner agreed with applicants that Jain "supports the use of monoclonal antibodies for targeting solid tumors." Action at page 11. The Examiner alleged, however, that Jain "does not enable the claimed invention of treating HER2 overexpressing cancer in a patient comprising administering mAb74 or any antibody that binds HER2 and induces apoptosis because Jain only teaches the advantage of antibodies in localizing to tumors and does not teach the successful treatment of cancer using the claimed antibodies or any related antibodies." *Id.* at pages 11 and 12.

Applicants have not relied on Jain to enable the present claims. Rather, applicants argued in the Response filed February 2, 2006, that Jain does not refute a finding that the present claims are enabled. In fact, Jain teaches that antibodies can be used to specifically target tumor cells without harming normal tissue. Jain at page 64, right column, first full paragraph. Applicants are unsure on what basis the Examiner continues to cite Jain as evidence that the present claims are not enabled. Applicants

respectfully request that the Examiner indicate which specific passages in Jain are currently relied on for the Examiner's contention that present claims are not enabled.

Herceptin, Stancovski, Lewis, and U.S. Patent No. 5,677,171

The Examiner alleged that

> the success of Herceptin as a treatment of HER2-overexpressing cancer is an example of only one of many anti-HER2 antibodies in the art that was successful for treating cancer [in] the clinical setting, which exemplifies the unpredictability of anti-HER2 antibodies in their ability to treat cancer.

Action at page 12.

Applicants respectfully traverse. The Examiner has presented no evidence that "many anti-HER2 antibodies in the art" were even tested for treating cancer in a clinical setting. Rather, the Examiner merely points to Herceptin, and then apparently contends that because many other antibodies exist, Herceptin must be the only antibody that was successful in a clinical setting. Applicants assert that the Examiner must rely on specific evidence to support a finding of a lack of enablement. Applicants remind the Examiner that, according to the MPEP, "[t]he Examiner should **never** make the determination based on personal opinion. The determination should always be based on the weight of all the evidence." MPEP § 2164.05 at 2100-198 to 199 (emphasis in original). Thus, applicants request that the Examiner provide evidence to support the contention that many anti-HER2 antibodies were tested in the clinical setting, but only Herceptin was successful.

The Examiner further alleged that

> Stancovski, Lewis, and US Patent 5,677,171 exemplify the unexpected nature and function of HER-2 binding antibodies by teaching that some HER2 antibodies actually accelerate tumor growth and that not every HER2 antibody

> can be used to effectively treat cancer as Herceptin (see the Office Action mailed September 22, 2005, p. 15). The art teaches that HER2 antibodies have different and sometimes unexpected functions, and it cannot be predicted which ones will effectively treat cancer. The specification discloses that mAb74 has the unexpected result of inducing apoptosis.

Action at pages 12 to 13.

Applicants respectfully traverse. Applicants assert that the fact that some anti-Her2 antibodies may not induce apoptosis in Her2 overexpressing cells does not impact the present analysis. Applicants have enabled a method of selecting antibodies that bind Her2 and induce apoptosis in Her2 overexpressing cells. That method will exclude antibodies that lack those activities, including, for example, antibodies that accelerate tumor cell growth.

Stancovski discusses five anti-Her2 antibodies that were selected *solely for their ability to bind to Her2 on the surface of cells*. *See* Stancovski at page 8692, right column, second full paragraph. Stancovski then tested those antibodies for inhibition of cell proliferation *in vitro* and inhibition of tumor growth *in vivo*. *See, e.g.*, Stancovski at page 8694, Table 1. The two antibodies that showed the most inhibition of cell proliferation *in vitro*, N12 and N29, also showed the greatest inhibition of tumor growth *in vivo*. *Id.* Thus, Stancovski actually supports applicants' contention that *in vitro* activity is a reasonable predictor of *in vivo* activity.

Furthermore, applicants assert that Stancovski fails to support the Examiner's contention that Stancovski "exemplif[ies] the unexpected nature and function of HER-2 binding antibodies." Rather, Stancovski supports a finding that the present claims are enabled. As discussed above, applicants assert that the present enablement inquiry

can be divided into two separate questions: first, whether the recited antibodies that bind Her2 and induce apoptosis in Her2-overexpressing cells are enabled; and second, whether a method of treating cancer characterized by Her2 overexpression in a patient is enabled. The Federal Circuit in *Wands* squarely held that it is routine in the antibody arts to make antibodies to a desired antigen *and screen those antibodies for desired characteristics*. Thus, the recited antibodies that bind Her2 and induce apoptosis in Her2 overexpressing cells are enabled, following *Wands*.

The second inquiry is whether a method of treating cancer characterized by overexpression of Her2 in a patient is enabled. Applicants assert that one skilled in the art, using the teachings in the specification and the knowledge in the art at the time of filing, could have practiced such a method without undue experimentation. As discussed above, applicants assert that one skilled in the art would conclude that there is a reasonable correlation between the *in vitro* results obtained with the NCI Panel and expected *in vivo* results. Furthermore, Stancovski supports that conclusion. Stancovski screened the anti-Her2 antibodies for inhibition of cell proliferation. *See* Stancovski, Table 1. Selecting only those antibodies with the desired characteristic, *i.e.*, inhibition of cell proliferation, the results of the tumor growth inhibition test in Stancovski demonstrate a good correlation between the *in vitro* activity of the selected antibodies and their *in vivo* activity. *See* Stancovski, antibodies N12 and N29 in Figure 1. Thus, Stancovski supports applicants' assertion that a reasonable correlation exists between the *in vitro* activity of the recited antibodies and their *in vivo* activity, for example, in the claimed methods.

Applicants assert that Lewis does not "exemplify the unexpected nature and function of HER-2 binding antibodies." In fact, Lewis teaches that there is generally a good correlation between Her2 expression in tumor cell lines and antibody-induced growth inhibition by anti-Her2 antibodies. Lewis at page 259, right column, first full paragraph, and page 260, Figure 3. The present claims recite a method for treating cancer characterized by overexpression of Her2, and applicants assert that Lewis teaches that anti-Her2 antibodies are particularly effective against tumor cell lines that overexpress Her2.

Furthermore, Lewis only tested the anti-Her2 antibodies *in vitro.* As applicants have discussed above, it was routine in the art at the time of filing to make antibodies to a desired antigen (*i.e.*, Her2) and screen them for desired properties (*i.e.*, the ability to induce apoptosis in Her2 overexpressing cells), following *Wands*. Lewis does not alter that conclusion. In fact, Lewis carried out the type of screen that was found to be routine in *Wands*; Lewis selected antibodies that bound to a desired target (*i.e.*, Her2) and tested them for a desired activity (*i.e.*, growth inhibition).

Lewis has no bearing on the second inquiry in the present enablement analysis: whether the use of those antibodies in a method for treating cancer characterized by overexpression of Her2 in a patient is enabled. Lewis never attempted to use the anti-Her2 antibodies *in vivo*. Thus, while Lewis demonstrates that it is within the skill in the art to make an antibody to a selected antigen and test it for a desired property (*i.e.*, inhibition of cell proliferation), Lewis presents no experiments to address the correlation between that *in vitro* activity and *in vivo* activity, nor does Lewis address whether one

skilled in the art could use the selected antibodies *in vivo* without undue experimentation. Lewis simply never attempted to do so.

Applicants assert that U.S. Patent No. 5,677,171 (Hudziak) also does not "exemplify the unexpected nature and function of HER-2 binding antibodies." As discussed above, Applicants assert that one skilled in the art could make an antibody or fragment thereof that binds to Her2 and induces apoptosis in Her2 overexpressing cells using the teachings of the specification and the knowledge in the art without undue experimentation. Discarding some undesirable antibodies in the selection process is simply part of the experimentation permitted by *Wands*. Thus, applicants assert that Hudziak has no bearing on whether the present claims are enabled.

Strobel

The Examiner alleged that Strobel teaches that "although antibodies share the same binding properties, they may not predictably both share the same function, for example, the function of treating a patient with cancer." Action at page 14.

Applicants respectfully traverse. Applicants assert that nowhere does Strobel discuss a correlation between *in vitro* activity of an antibody and *in vivo* activity of an antibody, much less whether two antibodies may "predictably both share the same function, for example, the function of treating a patient with cancer." Rather, Strobel discusses two antibodies that *have different functions* in vitro, so applicants assert that one skilled in the art would also predict that those antibodies also have different functions *in vivo*.

Strobel discusses two antibodies, antibody 4B4, which is ineffective at neutralizing cell binding to mesothelium, and antibody MAB13, which is effective at neutralizing cell binding to mesothelium. Strobel at page 366, left column, second full paragraph. As applicants have discussed above, it was routine in the art at the time of filing to make antibodies to a desired antigen and screen them for desired properties, following *Wands*. Such a process would exclude antibodies that lack the desired activity. Strobel does not alter that conclusion. In fact, Strobel carried out the type of screen that was found to be routine in *Wands*; Strobel selected antibodies that bound to a desired target (*i.e.*, β1) and tested them for a desired activity (*i.e.*, neutralizing cell binding to mesothelium).

Applicants assert that Strobel has no bearing on the second inquiry of the present enablement analysis. Strobel never attempted to use the anti-β1 antibodies *in vivo*. Thus, while Strobel demonstrates that it is within the skill in the art to make an antibody to a selected antigen and test it for a desired property, Strobel presents no experiments to address the correlation between the *in vitro* activity of the antibody and the *in vivo* activity, nor does Strobel address whether one skilled in the art could use the selected antibodies *in vivo* without undue experimentation. Strobel simply never attempted to do so.

For at least the reasons discussed herein, Applicants assert that the specification enables the present claims. Applicants respectfully request reconsideration and withdrawal of the enablement rejection under 35 U.S.C. § 112, first paragraph.

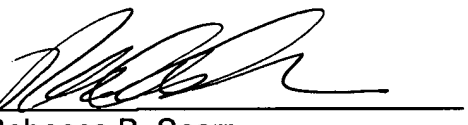
Applicants assert that the application is in condition for allowance and respectfully request that the Examiner issue a timely Notice of Allowance. If the Examiner does not consider the present application to be allowable, the undersigned requests that, prior to taking action, the Examiner call her at (650) 849-6656 to set up an interview.

Please grant any extensions of time required to enter this Response and charge any additional required fees to Deposit Account No. 06-0916.

Respectfully submitted,

FINNEGAN, HENDERSON, FARABOW,
GARRETT & DUNNER, L.L.P.

Dated: August 21, 2006

By: 

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Cytokine Expression and Networks in Rheumatoid Arthritis: Rationale for Anti-TNFα Antibody Therapy and Its Mechanism of Action

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ABBREVIATIONS:

TNFα	tumour necrosis factor
RA	rheumatoid arthritis
IL-1	interleukin-1
IL-10	interleukin 10
GM-CSF	granulocyte macrophage colony stimulating factor

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The cloning of cytokine cDNAs has permitted the analysis of cytokine expression in diseased sites such as rheumatoid joints. A very wide range of cytokines were detected, mostly with proinflammatory activities. From the analysis of cytokine regulation in rheumatoid joint cell cultures using neutralizing anti-cytokine antibodies, it was found that blockade of TNFα reduced the production of other proinflammatory cytokines. Hence TNFα was a potential therapeutic target. This concept was tested successfully in collagen induced arthritis in mice and led to clinical trials of anti-TNFα antibody in rheumatoid arthritis (RA) in humans. The mechanism of action of anti-TNFα will be discussed. © *1996 Wiley-Liss, Inc.*

INTRODUCTION: RATIONALE FOR ANTI-TNFα THERAPY— DEFINITION OF TNFα AS A "PIVOTAL" CYTOKINE

Rheumatoid arthritis is an autoimmune disease characterised by chronic inflammation of the synovial joints leading to destruction and loss of function. Its aetiology is unknown [1]. Since cytokines are major mediators of immunity and inflammation, an augmented expression of cytokines in the synovium could be anticipated. With the development of more sensitive techniques of local cytokine evaluation, using cDNA probes, in situ hybridisation as well as bioassays, it was possible to assess synovial cytokine expression [e.g., 2–5]. (This has recently been reviewed in detail [6]). However, the results, while confirming the upregulation of cytokine expression, did not identify any pathogenic cytokines, since many were augmented. The plethora of proinflammatory cytokines detected led some workers to conclude that cytokines were not good targets for therapy, as it was impractical to block all of them (discussed in reference 7).

Our approach was to try to

determine whether some cytokines were more critical to the disease process, and hence could be targets for immunotherapy. The approach was to develop an in vitro model system of human rheumatoid arthritis by culturing dissociated synovial joint cells. Most prior studies of rheumatoid joint cells had attempted to study a pure population of "synoviocytes," derived by passaging the "fibroblast-like" cells and discarding the complex mixture of lymphocytes and macrophages which make up the predominant cells in the synovium.

Our culture system has features of the rheumatoid process. The dissociated cells reaggreate rapidly, presumably due to their upregulated expression of adhesion molecules, and produce proinflammatory cytokines at a high rate for the 5 or 6 days in which we studied these cultures [3]. As this in vitro system reproduced the cytokine production profile found in synovium, ex vivo, it was a system for analysing cytokine regulation in a diseased site, in vitro and for investigating why cytokine production was prolonged, unlike that found upon mitogenic stimulation of normal cells.

The approach taken was to neutralise potential signals using neutralising antibodies. Since IL-1 had been clearly documented as being a potent inducer of destruction of cartilage and bone, the major lesions in RA, we initially studied regulation of IL-1 production.

The results obtained were surprising. Instead of many signals each partially regulating IL-1 biological activity, blocking TNFα abolished IL-1 biological activity within 3 days [8]. This was the first clue that TNFα may be the "pivotal cytokine" in RA. The effectiveness of IL-1 downregulation in vitro after anti TNFα was added, suggested that the other proinflammatory cytokine in the joint may be regulated concordantly. Hence, the effects of anti-TNFα on GM-CSF, IL-6, and IL-8 production were investigated. All of these were markedly diminished by anti-TNFα. The kinetics of inhibition were slower, needing approximately 5 days for a marked effect. This suggested that IL-1 may need to be inhibited before GM-CSF, IL-6, or IL-8 levels are reduced [9,10]. This was evaluated using IL-1 receptor antagonist (IL-1ra). IL-1ra diminishes IL-6 and IL-8 production, but not the TNFα or IL-1 itself [10]. Taken together, these results suggest that the proinflammatory cytokines are linked together in a cascade as illustrated in Figure 1. The proinflammatory cytokine cascade thus resembles an electrical circuit in "series" and not in "parallel" as discussed previously [11]. Furthermore, the scheme illustrated in Figure 1 suggests that TNFα is a good target for therapy, as neutralizing it will concomitantly reduce the levels of other proinflammatory mediators. What this scheme does not clarify is what drives the production of TNFα in RA joints—it is a topic we are actively studying, without detailed success. We think it is likely to be a combination of signals, many of which come from T cells [12].

IS RHEUMATOID ARTHRITIS DUE TO A LACK OF ANTI-INFLAMMATORY CYTOKINES OR CYTOKINE INHIBITORS?

This is a reasonable hypothesis, and with the paucity of our knowledge of inhibitors, is not easy to exclude. We and others have analysed the expression of inhibitors and the evidence indicates that they are upregulated. This applies to IL-10 [13], soluble TNF receptors [14], and IL-1ra [15], but not to IL-4, which is weakly expressed.

IL-10 levels in RA synovial cultures vary widely [13], with most in the area of 500 pg/ml. The levels produced are biologically meaningful, as anti-IL-10 treatment augmented levels of IL-1 and TNFα by 2–3-fold within 24 h in RA joint cell cultures. It is conceivable that levels detected in supernatant do not fully reflect IL-10 production, as there is evidence for membrane IL-10, and there would be IL-10 consumption.

Soluble TNF receptors are produced by proteolytic cleavage of membrane receptors and neutralise TNF bioactivity. Soluble TNF-R levels are upregulated in RA. Serum levels of the p55 and p75 TNF-R are elevated 2–3-fold compared with normal sera [14]. More relevant are the results of paired serum and synovial fluid samples, with the latter 3–4-fold higher, indicating that much of soluble TNF-R may come from the joints. While there has been suggestions that soluble TNF-R can "protect" TNF, we do not believe this to be true in RA. Synovial fluid samples typically have no TNF bioactivity, despite ELISA detectable TNF. Adding antibodies to soluble TNF-R can unmask TNF lytic activity, thus demonstrating that in this context soluble TNF-R is neutralizing TNF [16]. IL-1ra levels are also upregulated in both the joints and serum of RA patients, but not sufficiently so since IL-1 is still biologically active [15,17].

The above results, taken together, make it clear that there is upregulation of both inhibitory

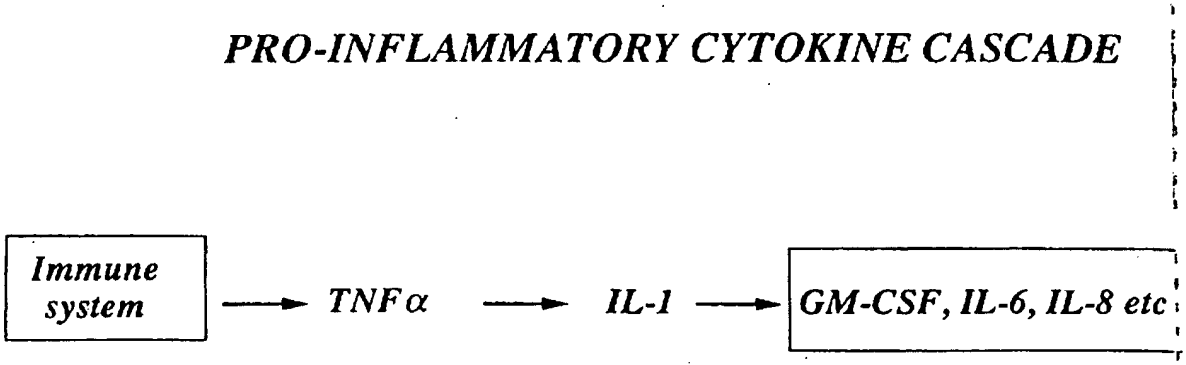

FIGURE 1
Pro-inflammatory cascade.

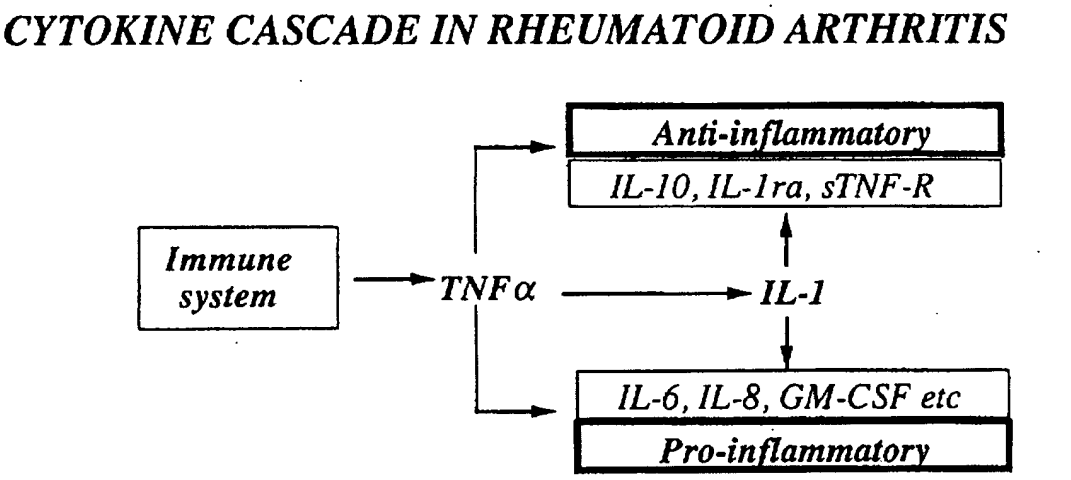

FIGURE 2
Cytokine cascade in RA. Reproduced with permission from: Feldmann, M., Brennan, F.M., and Maini, R.N. (1996) Rheumatoid Arthritis. Cell 85:307–310.

cytokines and cytokine inhibitors. A lack of their production is thus not a major etiological factor. However, there is a relative imbalance, in favour of the proinflammatory mediators, as based on direct evaluation, e.g., bioactive TNF and GM-CSF are produced from RA joint cell cultures [9,16]. Our concept is that there is a disregulated equilibrium, in favour of the proinflammatory cytokines (Fig. 2).

In view of the upregulated anti-inflammatory mediators, it was of interest to evaluate whether their production in RA tissue is dependent on the prior production of TNFα. Are these also linked in a cascade? This has been studied, IL-10 levels in RA cultures are diminished by anti-TNFα and IL-1ra, suggesting that both arms, pro- and anti-inflammatory are TNFα dependent (Fig. 3).

WHAT CAN BE LEARNED ABOUT THE PATHOGENESIS OF RA FROM CLINICAL TRIALS OF ANTI-TNFα

The major drive for the clinical trials of anti TNFα antibody in RA was to establish whether, as predicted, it was the pivotal cytokine of major importance in the pathogenesis and hence a useful therapeutic target. This was readily established, as the neutralizing antibody, cA2, used at high concentrations, rapidly induced a high

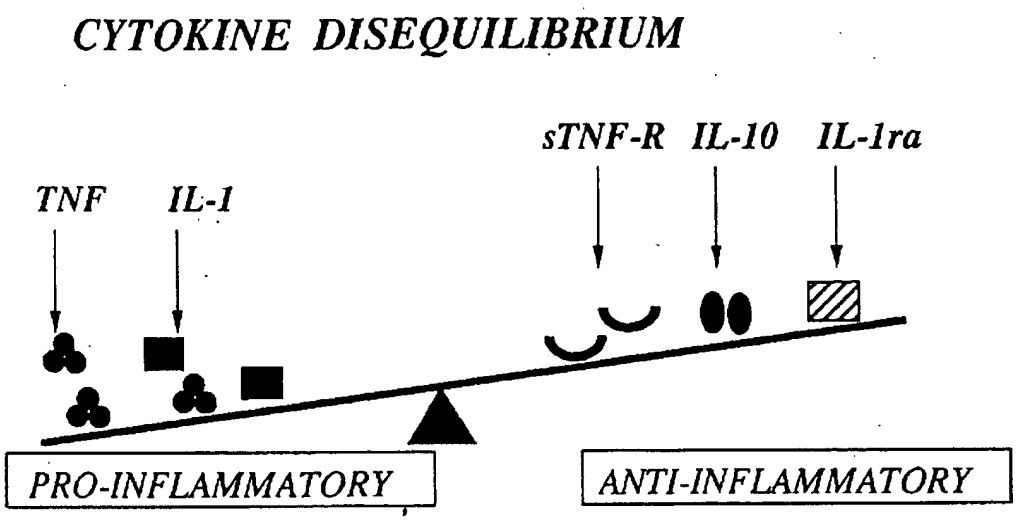

FIGURE 3
Cytokine disequilibrium. Reproduced with permission from: Feldmann, M., Brennan, F.M., and Maini, R.N. (1996) Rheumatoid Arthritis. Cell 85:307–310.

degree of clinical benefit, noted in all clinical parameters tested, and substantiated by rapid and marked changes in biochemical markers of inflammation [7]. Verification of this has come not only from our own placebo controlled studies [18] and our retreatment studies [19], but also the subsequent studies of other groups with another antibody [20] or an IgG TNF-R fusion protein [21].

One of the advantages of using a monoclonal antibody to analyse mechanisms of disease is that, unlike small organic chemicals, antibodies do not penetrate cells, and hence have more defined modes of action. With an organic chemical, it is not possible to be sure that any effect noted is a direct consequence of its known mechanism of action; it could be due to a secondary as yet unknown biochemical target. An example is RU486, which blocks multiple steroid receptors [22]. Thus, using anti-TNFα antibody, we have sought to gain insights into the pathogenesis of RA. One of the first studies was to confirm whether the analysis of the cytokine cascade in the rheumatoid joint was true in vivo.

IS THERE A PROINFLAMMATORY CASCADE IN RHEUMATOID PATIENTS IN VIVO?

Cytokines are chiefly "local" mediators, unlike hormones, which primarily travel to their destination via the blood [23]. Hence, it is not easy to verify this concept, as the readily available specimens serum has low levels of most cytokines, including IL-1. However, IL-6 is readily detectable in the circulation in a variety of pathologies, including RA, and IL-6 levels are rapidly diminished after anti-TNFα infusion [7]. This verifies that there is indeed a proinflammatory cytokine cascade in vivo, which has not been observed with other cytokines such as IL-1β, whose levels in RA serum are within the normal range [24].

IS THE PRODUCTION OF ANTI-INFLAMMATORY MEDIATORS DEPENDENT ON TNFα?

We are still in the process of investigating this question. However, clear-cut results have been obtained with IL-1ra [24] and soluble TNF receptors (unpublished). This confirms that TNFα drives the production of anti-inflammatory mediators in vivo, and may help explain why, although there is reasonably prolonged benefit, there are no cures, even though the equilibrium of cytokines has presumably been temporarily rectified.

WHY DOES THE CLINICAL BENEFIT OF cA2 OUTLIVE NEUTRALIZING LEVELS IN THE BLOOD?

Depending on one's point of view, anti-TNFα therapy is either short- or long-lived. It is short-

lived in the sense that there are no cures. All patients treated so far have relapsed. However, the benefit is longer than may have been expected, considering the half-life of chimaeric antibodies. A comparison of the pharmacokinetics with the Paulus 20% response [25] reveals that at all doses (1, 3, 10 mg/kg) the Paulus 20% response in approximately half of all individuals outlasts the neutralizing blood levels. Why is this so?

It is known that TNFα is a major inducer of endothelial adhesion molecules, such as E selectin and ICAM-1 [26]. TNFα is also an inducer of chemokines, as discussed above for IL-8. Hence, TNFα could be having a major effect on the recruitment of leucocytes to the joints, by influencing both arms of the recruitment cascade, adhesion, and chemotaxis. A variety of analyses on the serum of patients in the anti-TNFα clinical trials has provided evidence which supports the possibility that leucocyte trafficking is diminished. The evidence includes a rapid augmentation in lymphocyte counts occurring soon after anti-TNFα infusion, diminution of serum soluble E selectin, and diminution of serum soluble ICAM-1. If only patients which received anti-TNFα at 1 mg or 10 mg were analysed (placebo group excluded), there was a good correlation between benefit (20% Paulus response) and changes in lymphocyte counts, soluble E selectin, and ICAM-1 [27]. Supporting evidence comes from immunohistological analysis of joint biopsies before and after anti TNFα infusion.

It was found that joint cellularity is reduced and endothelial E selectin is reduced, as well as various markers of inflammation. This data were obtained after rigorous attempts at standardisation, and represent the concordant results obtained by two "blinded" observers [28]. A clinical trial to verify changes in trafficking, using radiolabelled WBC, is in progress.

CONCLUSIONS

The above studies have illustrated many aspects of research into the pathogenesis of a complex multifactorial disease. First, it is possible to use cytokine analysis and regulation to provide good therapeutic targets which are verifiable in clinical trials. The success of anti-TNFα in RA prompted equally successful clinical trials in Crohn's disease [29,30] which established in that disease that TNFα was also the "pivotal" cytokine. This is an example of cross-fertilisation at the clinical level. There are likely to be a number of diseases in which anti-TNFα therapy will be beneficial, e.g., multiple sclerosis, but it is unclear if in that example the benefit will be as dramatic with an antibody, as it will not penetrate the blood brain barrier effectively once inflammation in the CNS is reduced. Other anti-TNF modalities may be more effective, e.g., rolipram [31,32].

Secondly, antibodies are a very useful way of testing therapeutic concepts. The same, or more often related, antibodies may be used for in vitro analysis, in vivo animal models, as well as clinical trials.

Third, considerable insights into the pathogenesis of complex disease may be obtained by analysing data from clinical trials, if it can be "guaranteed" that the agent used has a defined mode of action, as occurs with a monoclonal antibody. We do not yet know what is the full range of important actions of TNFα in the pathogenesis of RA, but driving the pro- and anti-inflammatory cascades and driving leucocyte trafficking are two of the more important effects.

But perhaps the most important consequence is to encourage future studies, in RA to determine how to convert improvement into remission, and in other diseases to provide testable therapeutic targets.

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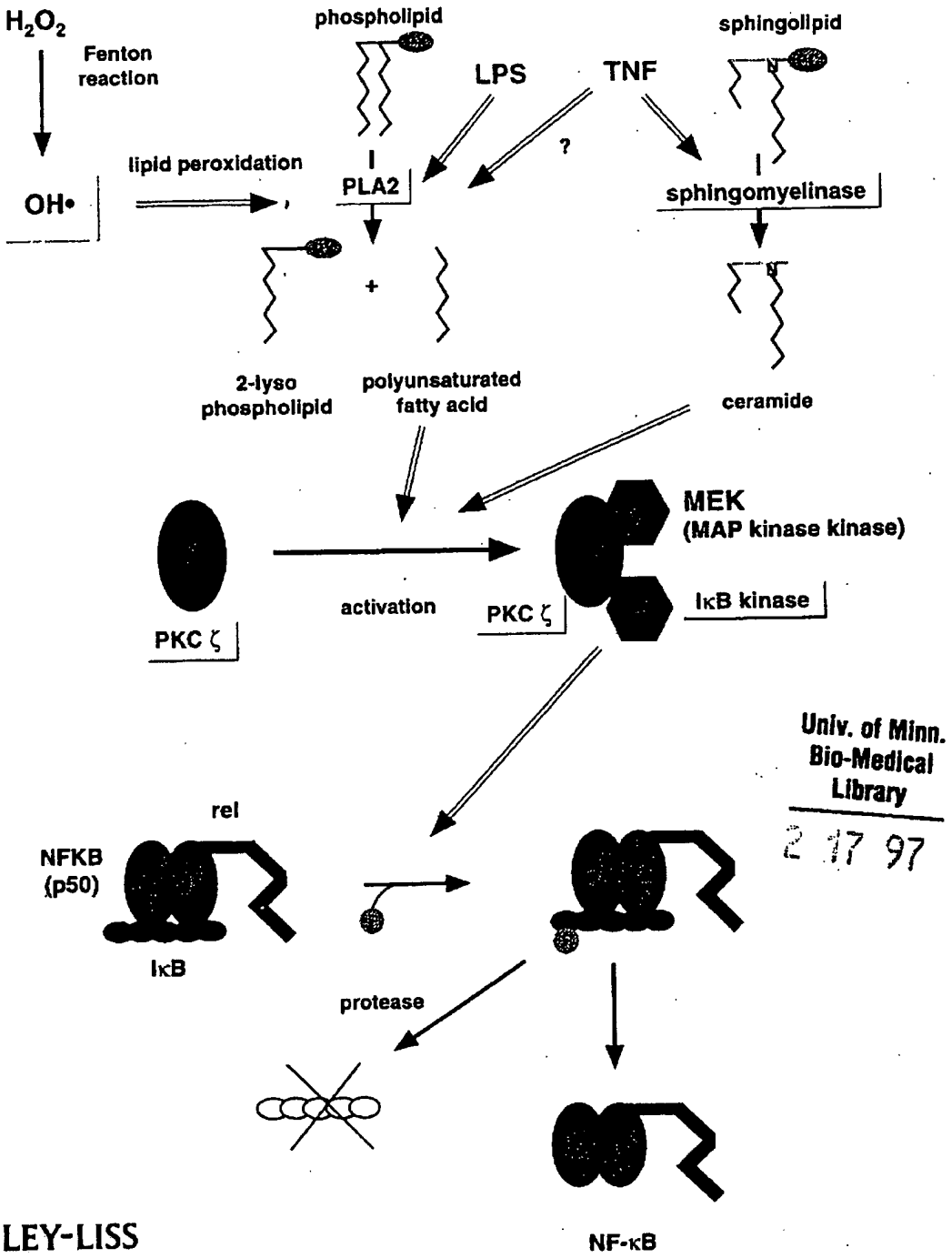

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Monoclonal anti-TNFα Antibody as a Probe of Pathogenesis and Therapy of Rheumatoid Disease

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THE BURDEN OF RHEUMATOID DISEASE AND LIMITATIONS OF CURRENT THERAPY

Rheumatoid arthritis (RA) is a chronic disabling and painful disease of multiple joints which is prevalent in all parts of the world. Population studies suggest that around 1% of the population is affected, the disease being three times commoner amongst women than men. The peak incidence is in middle age but no age is exempt and it afflicts all age groups including children and the elderly. Although the disease spectrum is broad, ranging from mild to severe, the disabling effects are cumulative and, in the severe group, associated with premature death (Erhardt et al. 1989, Pincus & Callahan 1993).

Current management of RA is based around a multidisciplinary team with drug therapy playing a key role in alleviating symptoms, maintaining mobility and slowing the progression of joint damage. However, a great majority of patients require frequent changes in drug therapy due to loss of efficacy and/or to toxicity and the lack of significant control of disease becomes all too self-evident in the second decade from onset. Surgical treatment, including multiple joint prostheses, provides respite but highlights the failure of medical treatment. The economic, social and personal cost of RA is clearly unacceptable and motivates the search for better and more effective therapeutic and preventative measures.

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NEW THERAPIES BASED ON TARGETING DISEASE MECHANISMS

Initiating factors and cellular targets

Attempts at discovery of effective novel cures for RA are based on the premise that knowledge of the causal factors and pathogenesis of RA has advanced significantly enough to construct hypotheses which define cellular and/or molecular targets. The etiology of RA is largely unknown, although population and family studies, together with observed concordance rates of disease in monozygotic twins, indicate that multiple germline genes are implicated with the genetic contribution to RA of 15% (Silman et al. 1993); however, a recent re-analysis has suggested that genetic factors could account for up to 60% of susceptibility (Macgregor & Silman 1994). The polymorphic HLA-DR β chain genes (some DR4 subtypes and DR1), encoding the 'shared epitope' pentapeptide sequence QRRAA, appears to be the main identified susceptibility gene (Gregersen et al. 1987). However, in the majority of patients a major role is attributed to non-genetic factors, and it is suggested that environmental factors, sex hormones and function of the hypothalamic-pituitary-adrenal axis, play a part (Reviewed in Silman & Hochberg 1993, Masi 1994, Chikanza et al. 1992). The association of RA with a HLA-DR molecule, which is involved in antigen binding and presentation to T cells, the presence in rheumatoid joints of activated memory T cells, and of autoantibodies in serum of patients, has suggested an important role for an immune response in initiating and maintaining RA. However, no convincing evidence for a rheumatoid specific antigen has been forthcoming, although antigens such as collagen II, abundant in cartilage, are possible candidates, and do elicit an antibody response in a majortity of DR4 positive RA patients (Ronnelid et al. 1994). Indeed we have documented a single RA patient in whom collagen II specific T-cell clones were repeatedly cloned from joints over a period of several years (Londei et al. 1989), but, in general, we can only demonstrate T-cell reactivity to collagen II antigens in a minority of RA patients. Alternatively, analysis of T-cell receptors in the joints in some studies has pointed to a restriction and selection bias which could suggest the involvement of superantigens (Paliard et al. 1991).

Since the involvement of T cells is central to the immunological paradigm, a great deal of attention has been paid to characterizing the prominent T-cell infiltrate in RA joints. The results of these investigations have led to the general conclusion that $CD4^+$ cells of the memory phenotype which bear activation markers are predominant (Panayi et al. 1992). Although there is a lack of consensus on the level of T cell cytokine expression, with the majority of investigators only finding low, but detectable, levels of interleukin-2 (IL-2) and interferon γ (IFNγ) at protein level (Firestein & Zvaifler 1990, Buchan et al. 1988a), there is an emerging consensus that there is a deficiency of IL-4 secretion in established disease (Moissec et al. 1990, Simon et al. 1994). However, at mRNA level IFNγ

is consistently detectable (Buchan et al. 1988a, Cohen et al. in press), suggesting a predominance of Th1 over Th2 subsets of T cells (Mossman & Coffman 1989), compatible with a T cell driven chronic inflammatory disease.

These concepts of the etiopathogenesis of RA have suggested many therapeutic approaches, summarized in Table I. These range from proposals that manipulating sex hormones or supplementing corticosteroids to restore the hypothalamic-pituitary-adrenal axis may be beneficial, to seeking synthetic designer peptides which effectively 'block' the HLA antigen binding site thus preventing its occupation by a putative RA specific autoantigen. For those investigators convinced that collagen II is an important autoantigen, attempts are being made to define immunodominant or cryptic epitopes that might induce tolerance either by systemic administration or via the oral route (Trentham et al. 1993). Recent demonstrations that with substitutions of one or two amino acids, immunogenic peptides are converted into antagonists, which result in immunological unresponsiveness

TABLE I
Targeting initiating factors and the T cell response in RA

Target	Observation	Possible therapy
Sex hormones	Female preponderance Oral contraceptives protective	Anti-oestrogens, male sex hormones Progesterones
Hypothalamic-pituitary-adrenal axis	Disturbed, poor corticosteroid response to surgical stress	Early use of corticosteroids
HLA DR4/1	Association of 'shared epitope' with severe RA	Peptide blockade
Antigen	B and T cell collagen II reactivity	Induce tolerance by altered peptide ligands or soluble immunodominant peptides
Superantigen	Restricted TcR usage with CDR3 diversity	Induce tolerance with superantigens
Th1 cells	Predominant Th1 cell in joints is pro-inflammatory; IL-4, IL-12 anti-inflammatory and deviate to protective Th2 response	IL-4 (IL-10) therapy
T cells	Activated, $CD4^+$ memory cells enriched in synovium	IL-2 diphtheria toxin conjugate and monoclonal antibody therapy, e.g., anti-IL-7, anti-CDW52, anti-CD4, anti-CD5
Gut associated lymphoid system	Oral collagen II induces bystander suppression via TGF-β producing cells trafficking to joints	Oral collagen feeding

or induce secretion of anti-inflammatory cytokines (e.g., IL-4, IL-10), have raised hopes that peptide therapy may prove useful in RA (Evavold et al. 1993). Peripheral tolerance, under certain circustances, can also be induced by superantigens (O'Heir & Lamb 1990). Other manipulations with regulatory cytokines can convert a predominantly pro-inflammatory autoimmune Th1 response to an anti-inflammatory Th2 response, for example by administration of IL-4, with beneficial results (Racke et al. 1994).

Whilst many of these ideas are in early stages of development, T cell targeted therapies have already been used in clinical trials in RA. The major therapeutic modality has been the use of monoclonal murine and chimeric anti-CD4 antibodies on several hundred patients; additionally, a monoclonal antibody anti-CD5-toxin, murine anti-CD7 monoclonal antibody, a humanized anti-CDW52 monoclonal antibody (CAMPATH-1H) and an IL-2 diphtheria fusion toxin have all been used in clinical trials (reviewed by Elliott & Maini 1994). However, encouraging data from early open-label studies have not withstood more rigorous randomized placebo-controlled trials with disappointing results reported for anti-CD4, anti-CD5 immuno-conjugate and IL-2 diphtheria toxin fusion proteins (Moreland et al. 1993a, Van der Lubbe et al. 1994, Olsen et al. 1994, Moreland et al. 1993b). Progress with further testing of the promising results with CAMPATH-1H (Isaacs et al. 1992) has been set back by reports of susceptibility to virus and bacterial infections in treated patients.

Attempts at inducing antigen-specific regulatory T cells have been put to clinical trial by daily oral feeding of collagen type II, a constituent of cartilage, to RA patients in order to induce 'by-stander' suppression of activated T cells in joints (Trentham et al. 1993). In this approach it is speculated that oral xenogeneic collagen II activates a population of immunoregulatory gut associated lymphoid cells, which home to the joints, and there receive further activation signals from antigen-presenting cells which have locally processed collagen II from damaged cartilage. Here, anti-inflammatory cytokines such as TGF-β produced by the Th2, lymphocytes originating from gut, suppress pro-inflammatory Th1 cells (Chen et al. 1994). Promising results have been reported in early trials with some patients showing remission of their RA.

Cytokines and anti-cytokines

In our research, we have focused our attention on cytokines as promising therapeutic targets, since in the past decade impressive progress has been made in isolating a number of cytokines and their cognate receptors, and interactions between them which mediate the biological and pharmacological functions relevant to the pathogenesis of RA (Feldmann et al. 1993). For example, cytokines provide a framework for understanding how circulating leukocytes might adhere to and migrate into inflammatory sites, of which the RA joint is an example. At

these inflammatory sites cytokines regulate growth, differentiation, cell death and cellular interaction via autocrine and paracrine activities which are implicated in sustaining chronic immune and inflammatory reactions. Here cytokines also orchestrate interactions between the immigrant cells and locally resident connective tissue cells and matrix to produce adherent pannus tissue, which erodes cartilage and bone, with incomplete attempts at regeneration and repair.

In terms of specific cytokines found in RA joints, research from a number of latoratories, including ours, leads to the conclusion that many of the cytokines are abundantly expressed during both the chronic and acute stages of disease, even in patients receiving 'optimal' anti-rheumatoid therapy. These abundantly expressed cytokines include IL-1α, IL-1β and tumor necrosis factor (TNFα) which exert pleiotropic, overlapping and synergistic biological effects relevant to the joint pathology of RA (Buchan et al. 1988b). Relevant biological effects include induction of adhesion molecules such as E-selectin, intercellular adhesion molecule (ICAM-1) and vascular cell adhesion molecule (VCAM-1) which promote cell migration and adhesion in joints (Bevilacqua 1993), induction of inflammatory and tissue-damaging molecules such as prostaglandins and collagenases (Dayer et al. 1985); nitric oxide (Palmer et al. 1993); and effects on cellular function, such as activation of osteoclastic resorption of bone, and suppression of anabolic activity of chondrocytes and osteoblasts, thereby impairing regeneration of cartilage and bone (Saklatavala et al. 1985, Gowen et al. 1983). Other locally produced cytokines with relevant pro-inflammatory activity are chemokines, such as IL-8 (Brennan et al. 1990b) and RANTES (Schall 1991), which attract polymorphs and macrophages to joints; and GM-CSF which mobilizes and activates granulocytes and macrophages from the bone marrow, as well as mediating local effects, such as upregulating HLA Class II expression and interleukin-1 production, all of which ultimately promote inflammation. IL-6 is also over-produced and is important in inducing acute phase proteins by hepatocytes (Hirano et al. 1988). Its local action on differentiation of B cells may contribute to production of antibodies (hence immune complexes) which are pro-inflammatory, a proposition consistent with clinical benefit attributed to the use of intravenous anti-IL-6 antibody (Wendling et al. 1993). Therefore, blocking the activity of individual cytokines found in RA joints, i.e. IL-1, TNFα, IL-8, RANTES, GM-CSF and IL-6 might be expected to result in anti-rheumatoid effects, but the prospect of developing specific molecular inhibitor drugs for each cytokine is a daunting task, and fortunately subsequent events have shown it to be unnecessary (*vide infra*).

An appealing alternative therapeutic approach is based on exploiting the production of naturally occurring inhibitors of cytokines produced in RA joints. RA inflammation is characterized by enhanced, but insufficient, production of interleukin-1 receptor antagonist (IL-1ra) and soluble TNF receptors, which are the specific inhibitors of IL-1 and TNFα respectively (Deleuran et al. 1992a, Cope

et al. 1992), and of IL-10 (Katsikis et al. 1994), which inhibits synthesis of IL-1 and TNFα. Augmenting inhibitor production *in situ* or supplementing inhibitor by injecting recombinant soluble receptors, usually as multimeric-immunoglobulin fusion proteins, in order to increase affinity and biological half-life *in vivo*, provide further scope for anti-cytokine drug development.

PIVOTAL ROLE OF TNFα

Our hypothesis that TNFα is a potential target for therapeutic intervention in RA was derived from experiments performed *in vitro* on RA synovial tissue. These studies indicated that dissociated synovial tissue mononuclear cells (MNCs) from RA patients were highly activated, and if placed in culture would produce cytokines spontaneously for 5–6 days without exogenous stimulation. The cytokines which are produced in abundance include IL-1α and IL-1β (Buchan et al. 1988b), TNFα (Brennan et al. 1989), IL-6 (Hirano et al. 1988), IL-8 (Brennan et al. 1990b), TGFβ (Brennan et al. 1990a), GM-CSF (Haworth et al. 1991) and, as shown more recently, IL-10 (Katsikis et al. 1994). As IL-1 and TNFα have pro-inflammatory potential implicated in the pathogenesis of RA, we focused on the regulation of these cytokines.

Using a polyclonal anti-TNF antibody to block TNFα activity in the RA synovial cell cultures, we observed that this also resulted in the inhibition of IL-1 production (Brennan et al. 1989). The result suggested the presence of a 'cytokine cascade' within the synovium, with the prediction that blockade of a pivotal cytokine in this network would subsequently lead to blockade of other cytokines 'downstream'. This indeed was found to be the case in that the production of other pro-inflammatory cytokines including GM-CSF (Haworth et al. 1991), IL-6 and IL-8 (Butler et al. submitted) was also modulated by TNFα blockade. The biological effect of TNFα is enhanced further by our observation that both p55 and p75 surface TNF receptors are upregulated on RA synovial tissue cells (Brennan et al. 1991, Deleuran et al. 1992b) (Fig. 1). Indeed, CD68 positive macrophages at the cartilage pannus junction (Chu et al. 1992) also express abundant p55 and p75 TNF-R, indicating the potential of these cells to respond in an autocrine manner. The biological activity of TNFα is regulated to some degree, however, by the production of its native inhibitors soluble p55 and p75 TNF-R. These soluble TNF-R are also found in elevated amounts in RA plasma and in particular, in synovial fluid where their presence in excess neutralizes TNFα activity (Cope et al. 1992). The production of sTNF-R is insufficient however, as bioactive TNFα was found in all RA synovial MNC cultures tested.

A second immunoregulatory molecule, IL-10, is also abundantly produced by RA synovial MNC cultures (Katsikis et al. 1994). Endogenous IL-10 was found to be functional as its neutralization enhanced TNFα and IL-1 production 2–3 fold (Katsikis et al. 1994), indicating that as in the production of other cytokine

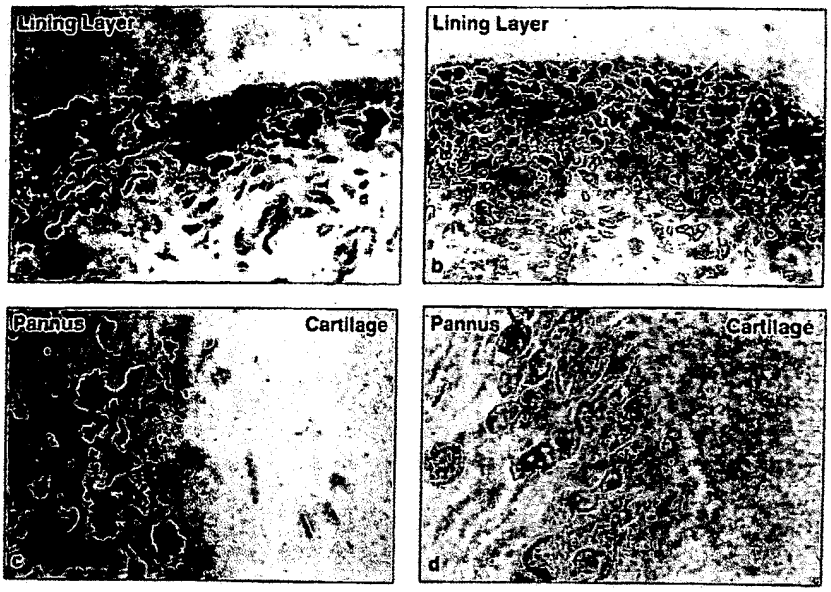

Figure 1. TNFα and p55 TNF-R demonstrated by immunohistology of synovium and cartilage-pannus junction in a RA joint. Majority of cells in synovial layer co-stain for TNFα (a) and TNF-R (b); similar co-localization of TNFα (c) and TNF-R (d) is observed at cartilage-pannus junction.

inhibitors including sTNF-R (Cope et al. 1992) and the IL-1ra (Deleuran et al. 1992a) homeostatic mechanisms are present in inflammatory environments, albeit in insufficient quantities, since cytokine production and the consequential pathological changes continue to occur.

EVIDENCE FOR THE IMPORTANCE OF TNFα FROM ANIMAL MODELS

Murine collagen-induced arthritis (CIA) has been extensively studied as a model for human rheumatoid arthritis (RA), principally because of the pathological similarities between the two diseases, including similar patterns of synovitis, pannus formation and erosion of cartilage and bone (Stuart et al. 1984). In addition, susceptibility to both human RA and murine CIA is linked to specific MHC class II genes, suggesting that an important step in the development of both diseases is the MHC-restricted activation of $CD4^+$ T cells (Holmdahl et al. 1989).

The CIA model has been used to elucidate pathogenic mechanisms of relevance to human disease and to identify potential targets for therapeutic intervention.

One of the methods used in such studies involves the administration of potentially pathological cytokines to naive mice or to mice previously immunized with type II collagen in order to demonstrate altered expression of disease. For example, the local administration, in recombinant form, of IFN-γ, IL-1β, or TNF to type II collagen-immunized mice has been shown to result in accelerated onset and increased severity of CIA, suggesting that these cytokines are involved in the induction of arthritis (Mauritz et al. 1988, Hom et al. 1988a, Thorbecke et al. 1992, Cooper et al. 1992, Brahn et al. 1992). None of the cytokines, however, was capable of initiating arthritis when injected into unimmunized mice, indicating that they could not, on their own, elicit a sustained inflammatory response. However, the experimental approach adopted in these studies has a number of limitations, including the fact that cytokines generally have extremely short half-lives *in vivo*.

An alternative approach would be to use transgenic mice to study the pathological effects arising from the sustained over-expression of cytokine transgenes. This approach powerfully demonstrated the arthritogenic potential of over-expression of TNFα in the joints of 3'-modified human TNFh-transgenic mice, which spontaneously develop a chronic arthritis that is prevented by anti-TNF treatment (Keffer et al. 1991). Our histopathological studies on the Kolias hTNF-transgenic mice show that the joints of these mice exhibit similar inflammatory lesions to those found in human RA, including proliferative synovitis and pannus formation. Most importantly, necrotic chondrocytes are oberved in the joints of hTNF-transgenic mice as well as severe focal erosions of sub-chondral bone (Fig. 2). These findings clearly show that the dysregulated expression of TNF is alone capable of inducing many of the pathological changes that are seen both in human RA and murine CIA, including full-blown destruction of cartilage and bone.

An alternative to the study of cytokines that contribute to the pathogenesis of arthritis is to treat collagen-arthritic mice or rats with specific anti-cytokine agents in order to show a reduction in disease severity. Following the identification of pro-inflammatory cytokines, in particular TNF and IL-1, in the joints of RA patients (Chu et al. 1991, Chu et al. 1992), we and others set out to address the question of whether the neutralization of such cytokines would lead to amelioration of autoimmune arthritis. A number of studies focused on the effect of anti-TNF treatment in CIA, partly because of data accumulated in our laboratories suggesting that, in RA at least, TNF was playing an important role in the induction of other pro-inflammatory cytokines. In CIA, two studies showed that monoclonal or polyclonal anti-TNF antibodies, administered before the onset of clinical arthritis, protected against the subsequent development of disease (Thorbecke et al. 1992, Piguet et al. 1992). Similarly, soluble TNF receptors, which are thought to serve as endogenous regulators of TNF activity, also protected against CIA when given during the pre-arthritic period (Piguet et al. 1992).

Our own research has focused largely on the effects of anti-TNF treatment in

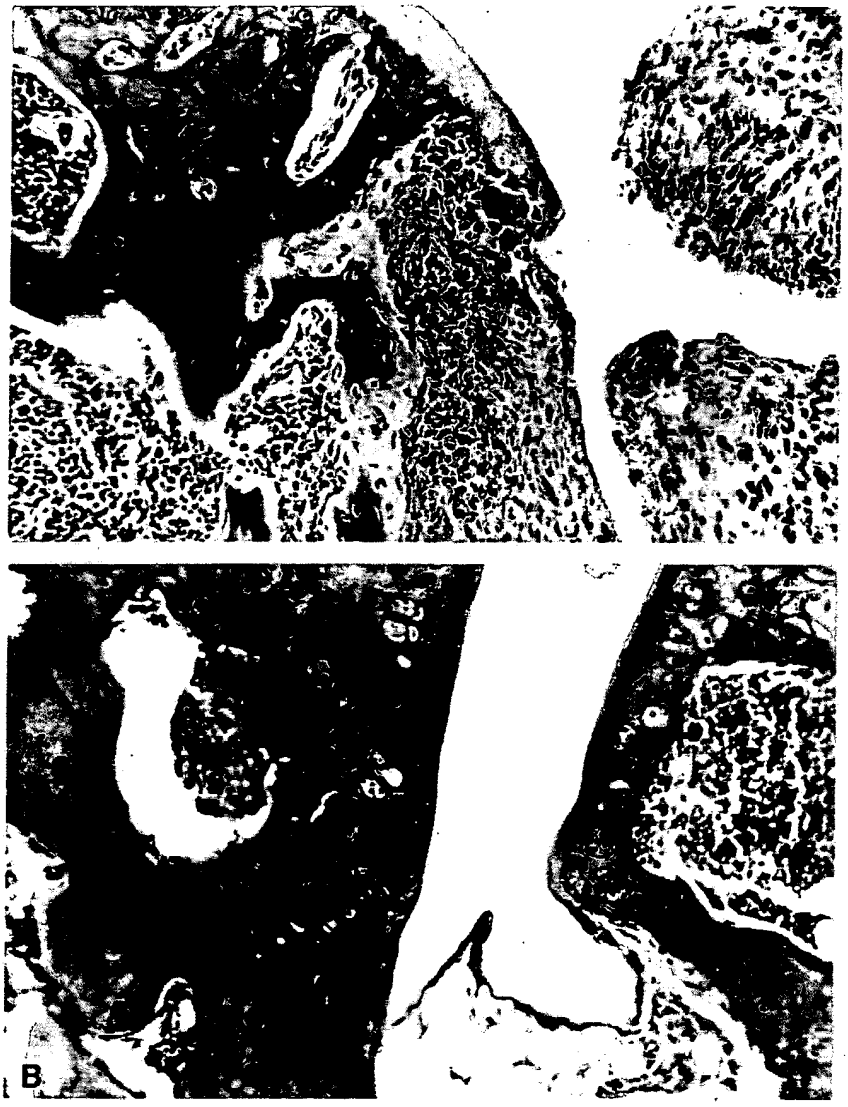

Figure 2. A: Light micrograph showing the cartilage-bone-pannus region of a knee joint from a hTNF-transgenic mouse. Note the focal erosion of sub-chondral bone by cellular pannus and loss of chondrocytes in cartilage. B: Normal joint and surfaces from non-transgenic littermate with chondrocyte-rich cartilage and normal bone.

established CIA, because of the greater significance of such studies in terms of human therapy. We were able to show that a hamster IgG1 anti-TNF monoclonal antibody (mAb) (TN3-19.12) administered over a 10-day period, starting immediately after the onset of clinically-detectable arthritis, caused a reduction in clinical score and suppressed paw-swelling, without altering circulating levels of anti-type II collagen IgG (Williams et al. 1992). In addition, histological analysis showed that anti-TNF treatment resulted in a significant reduction in joint erosion relative to control arthritic mice, as determined by the proportion of proximal-interphalangeal (PIP) joints showing erosive changes (Table I).

The long-term administration to man of anti-TNF mAbs containing epitopes of murine origin may be limited by the development of a neutralizing antibody response (Waldmann 1991). A TNF receptor-IgG fusion protein (of entirely human origin but containing neo-epitopes at the junction of IgG and TNF receptor) may, therefore, provide a less immunogenic alternative to mAbs, provided that the fusion protein can be shown to be effective *in vitro*. In one study, a human p75 TNF receptor-Fcγ fusion protein was found to reduce the clinical severity of CIA, whether administered before or after the onset of arthritis (Wooley et al. 1993a). We were subsequently able to confirm the clinical efficacy of this form of treatment in established CIA using a human p55 TNF receptor-IgG fusion protein. In addition, we found by histological assessment that treatment with p55 TNF receptor-IgG had resulted in a significant reduction in joint erosion (Table II) (Williams et al. 1995).

Studies to assess the effect of blocking IL-1 have also been carried out in CIA. Thus, IL-1ra and anti-IL-1β mAb both suppressed CIA when treatment was started before disease onset (Wooley et al. 1993b, Geiger et al. 1993). Furthermore, treatment after disease onset with polyclonal anti-IL-1α/β, or anti-IL-1β alone, profoundly suppressed both inflammation and cartilage destruction (Van den Berg et

TABLE II

Treatment	Dose	PIP joints with erosions
Saline	–	15/15 (100%)
Control mAb	300 μg	15/15 (100%)
Anti-TNF (TN3-19.12)	300 μg	9/15 (60%) P<0.05
Saline	–	11/12 (92%)
IgG control	100 μg	6/6 (100%)
TNF receptor-IgG (p55-sf2)	100 μg	6/12 (50%) P<0.05

Reductions in joint erosion following treatment of established CIA with anti-TNF mAb or p55 TNF receptor-IgG fusion protein. Both TNF-neutralizing agents were given three times over a 10 day period. At the end of the treatment period, sagittal sections of the PIP joints of the middle digits were studied in a blinded fashion for the presence or absence of erosions (defined as demarcated defects in cartilage or bone filled with inflammatory tissue). Based on original data published in Williams et al. (1992) and Williams et al. (1995).

al. 1994). It is clear, therefore, that IL-1 contributes significantly to the pathology of CIA. However, in the TNFα transgenic mouse, a disease model in which the arthritis is driven by TNFα, anti-IL-1 therapy also ameliorates disease (Kolias, personal communication). These data are consistent with our observations in RA, which demonstrate that TNFα regulates the production of IL-1, and is therefore a logical therapeutic target, although in CIA, unlike in RA, a TNFα-independent pathway of IL-1production may exist in addition.

The studies described above indicate that inflammatory cytokine-targeted therapy is effective in reducing the severity of CIA, and our work on anti-TNF treatment also indicates that the duration of the therapeutic effect is dependent on the presence of excess anti-TNF mAb in the circulation. However, results in patients are different, with the clinical benefit outlasting the 'therapeutic levels' of antibody by several weeks (unpublished data).

Experiments on animal models have provided further data that may be of relevance for the treatment of human disease. Early studies, for example, demonstrated that anti-CD4 mAb could block the induction of CIA if injected close to the time of immunization (Ranges et al. 1985), and this kind of study was used as evidence to support the initiation of clinical trials of anti-CD4 in human RA (Herzog et al. 1987). In CIA, however, anti-CD4 treatment was found to be ineffective when given *after* collagen immunization (Brahn & Trentham 1984), a finding that was confirmed in a subsequent study which showed that anti-CD4 treatment alone was ineffective in established CIA (Hom et al. 1988). Anti-CD4 treatment in human RA has been shown in a number of open clinical trials to provide a degree of transient clinical benefit to some patients (Herzog et al. 1989), although many patients fail to respond to therapy in spite of severe peripheral T-cell depletion (Burmester et al. 1992, Choy et al. 1992). Furthermore, the results of at least one placebo-controlled trial of anti-CD4 therapy in RA clearly showed a lack of clinical benefit (van der Lubbe et al. 1994) and there is, therefore, a degree of concordance between human RA and murine CIA with respect to the relative lack of efficacy of anti-CD4 treatment in established disease.

Having established the distinction in the efficacy of anti-TNF and anti-CD4 therapy after disease onset we carried out a study to determine whether a combination of T cell-targeted therapy and TNF-targeted therapy would have a more significant impact on CIA than either treatment alone. Thus, DBA/1 mice with established CIA were given intraperitoneal injections of either a depleting anti-CD4 (YTS191.1.2/YTA3.1.2) alone, anti-TNF (TN3-19.12) alone or anti-CD4 plus anti-TNF. The results showed that anti-CD4 m/Ab acted synergistically with both a sub-optimal dose (50 μg) and an optimal dose (300 μg) of anti-TNF mAb (Williams et al. 1994). The beneficial therapeutic effects of anti-CD4 and anti-TNF were observed both clinically, in the suppression of paw-swelling and in the prevention of new limb involvement and histologically, in the protection against joint erosion of cartilage and bone. Mice treated with anti-TNF alone developed

a non-neutralizing antibody response to the anti-TNF mAb which was completely blocked by concurrent anti-CD4 treatment, a finding that may be important in terms of long-term treatment in man.

We were subsequently able to provide evidence of synergy between anti-CD4 and TNF receptor-IgG fusion protein (Williams et al. 1995). As in the previous experiment, it was found that concurrent anti-CD4 treatment almost completely blocked the anti-TNF receptor-IgG fusion protein response, which in this experiment resulted in increased circulating levels of the fusion protein. One of the possible mechanisms, therefore, to account for the synergy between anti-CD4 and TNF receptor-IgG is the prevention of a neutralizing antibody response leading to increased half-life of the fusion protein. However, other mechanisms of synergy also exist because synergy was observed between anti-CD4 and anti-TNF mAb in the absence of significant changes in levels of free anti-TNF in the serum (Williams et al. 1994).

In conclusion, these studies have shown that anti-TNF treatment leads to a degree of amelioration of CIA that is significantly increased when combined with anti-CD4. It is possible that combination therapy, targeted at the autoimmune response as well as the inflammatory cytokine network will provide the basis for the future treatment of RA.

EVIDENCE FOR ROLE ON TNFα FROM CLINICAL TRIALS

Having gathered *in vitro* and *in vivo* evidence of an important role for TNFα, the concept that neutralizing doses of monoclonal anti-TNFα antibody administered systemically might suppress RA was finally tested in patients by clinical trials. The aims of these trials was firstly to establish proof of principle, i.e., that it was possible to demonstrate clinical efficacy of an anti-TNFα chimeric monoclonal antibody (cA2) by employing validated clinical and laboratory parameters and showing a dose-response effect; secondly, to assess whether the magnitude and duration of benefit were sufficiently significant to herald a therapeutic advance; and thirdly, to investigate the safety and tolerability of the chimeric monoclonal antibody. To date, results of three clinical trials which have been completed on 93 patients have clearly demonstrated a substantial benefit in the short term in 80–90% of patients, following intravenous injection of cA2, with excellent tolerability and few side-effects.

The first study was designed primarily to test our hypothesis that TNF was of importance in the pathogenesis of RA. In this pilot study, we recruited 20 patients from our own hospital clinic and following referral from outside physicians. Each patient had refractory disease, in that they had a relatively long disease duration (median of 10.5 years), had either failed therapy or had reacted badly to several standard disease modifying antirheumatic drugs (DMARDS) and had continuing evidence of active synovitis. Although patients were permitted to continue on

stable doses of non-steroidal anti-inflammatory drugs and low dose corticosteroids, any disease-modifying drugs which the patients were taking at screening were withdrawn over a 4-week period, prior to entry.

The antibody used in the study and in subsequent trials was cA2 (Centocor, Pa, USA) a chimerized (human IgG_1/mouse F_v) monoclonal antibody with specificity for human TNFα, with maintenance of high affinity binding (Knight et al. 1993). Although this was the first occasion that such an antibody had been used in rheumatic disease and the dosage required was unclear, we had preliminary evidence from the CIA model as a guide to the dose range (Williams et al. 1992). We administered either 2 or 4 infusions of cA2 over a period of 2 weeks, each infusion lasting 2 hours. The total dose delivered in each treatment regime was the same (20mg/kg).

The patients were followed using a number of different clinical and laboratory measures of disease activity and the overall response to treatment was assessed by means of the Paulus criteria*, a composite disease activity index incorporating six independent variables (Paulus et al. 1990) modified to accommodate the format of our data (Elliott et al. 1993). The results from this small pilot study were most encouraging. 19 of the 20 completed all scheduled infusions, with the one patient dropping out at the 2-week infusion point because of a concomitant illness (bronchitis). Patients showed marked improvement in all clinical measures, with the most rapid changes seen in the duration of morning stiffness and pain score and gradual but still marked improvements in the swollen and tender joint counts. Other clinical measures including the grip strength and the patient's assessment of response also showed significant improvement. The clinical improvements were supported by significant falls in laboratory measures of disease activity including

TABLE III

IL-6 and measures of the acute phase response after treatment with cA2

Time (days after treatment)	Measure			
	IL-6 (pg/ml)	SAA (mg/ml)	CRP (mg/l)	ESR mm/hour
0 (entry)	*60 (42, 104)	245 (127, 513)	40 (28, 67)	55 (24, 77)
1	14 (1, 43)	190 (61, 415)	26 (18, 57)	**ND
7	40 (8, 60)	58 (26, 148)	5 (2, 13)	26 (19, 43)
14	32 (1, 51)	80 (38, 220)	6 (2, 29)	27 (15, 52)
28	10 (2, 48)	118 (44, 300)	11 (4, 32)	23 (16, 57)
56	3 (0, 14)	105 (54, 140)	6 (3, 25)	30 (21, 55)

* Median (interquartile range) of up to 20 patients per point.
** Not done.

*Paulus criteria: Significant improvement in at least 4 of 6 variables, defined as:

- at least 20% improvement in continuous variables (tender and swollen joint scores, duration of morning stiffness, ESR).
- at least 2 grade improvement in patient's and observer's assessment of disease severity.

the acute phase proteins, serum amyloid-A (SAA), C-reactive protein (CRP) and the erythrocyte sedimentation rate (ESR; Table III). The response in each of these measures was rapid, with maximal improvement from day 7 after treatment. Significant falls were also seen in serum levels of IL-6, an important cytokine involved in the hepatic acute phase response. For many patients, serum IL-6 levels had normalized by day 1 after treatment and remained low for the duration of the study (Table III and data not shown). Serial changes in serum IL-6, SAA and CRP in a patient with a good response to cA2 are shown in Fig. 3 and demonstrate normalization of IL-6 levels in advance of the acute phase proteins. The data are consistent with the hypothesis that TNF blockade leads to inhibition of IL-6 synthesis, with subsequent down-regulation of hepatic acute phase protein synthesis. Other laboratory measures which demonstrate a trend towards improvement included the white blood cell and platelet counts and the hemoglobin.

Although these results were obtained in an open label, uncontrolled trial, the consistency and extent of the clinical improvements and the parallel changes in laboratory measures of the acute phase response suggested that the responses

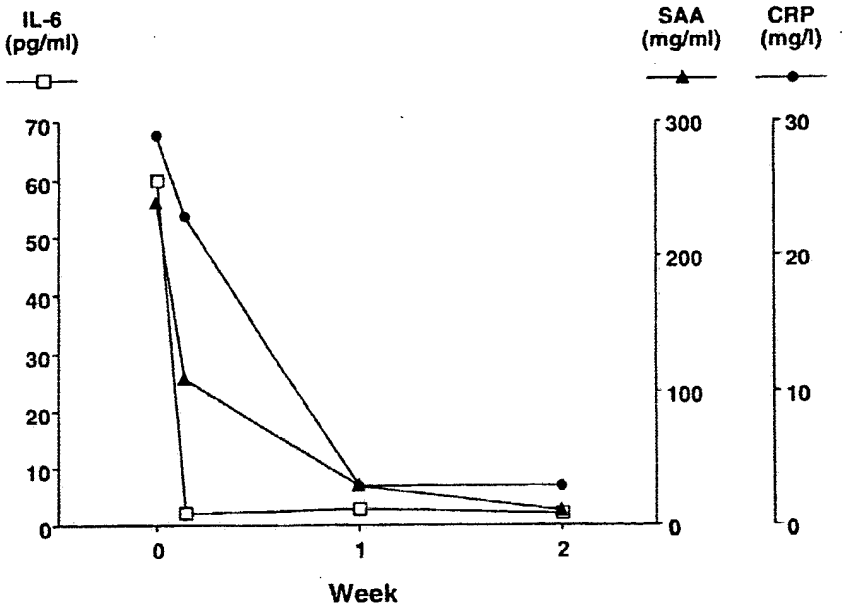

Figure 3. Changes in serum IL-6, SAA and CRP in a patient treated with cA2. The patient received a single infusion of 10 mg/kg cA2 at entry (week 0). IL-6 and SAA were measured by ELISA (Medgenix Diagnostics and Biosource International, respectively) and CRP were measured by rate nephelometry. Normal ranges: IL-6<10 pg/ml; SAA<10 mg/ml; CRP<10 mg/ml.

were likely to be due to a true therapeutic effect. As the definitive test of the importance of TNF in the pathogenesis of RA and of the clinical efficacy of cA2 in this disease, we devised a multi-center, randomized, double-blind study which compared the use of cA2 to placebo (human serum albumin) (Elliott et al. 1994a). Many of the design features, inducing the requirement for longstanding, refractory disease, the requirement for withdrawal from DMARD therapy and the need for stable doses of other concomitant medications were maintained into the 2nd study. Patients were given a single infusion of placebo or cA2 (at a dose of 1 or 10 mg/kg) and were followed by blinded observers for response.

The predetermined, primary endpoint of the study was the achievement at week 4 of a response according to the Paulus (20%) criterion. Using this measure there were substantial and clear differences between the 3 groups, with only 2 of the 24 placebo recipients (8%) responding compared with 19 of 24 patients (79%) treated with high-dose cA2. The responses of the low-dose cA2 recipients were intermediate, demonstrating a dose-response relationship (Fig. 4). If the threshold of improvement was raised to a 50% improvement in Paulus index at a 4-week end-point, then 58% of patients treated with the higher dose of cA2 gained benefits from treatment (Fig. 4). Although considered supportive in nature, the improvements in individual disease activity measures were also of interest and similar changes were seen in the cA2 treated groups to those seen in the open label study. In particular, the tender and swollen joint counts, the pain score, the duration of morning stiffness, the fatigue score, the grip strength and the patients'

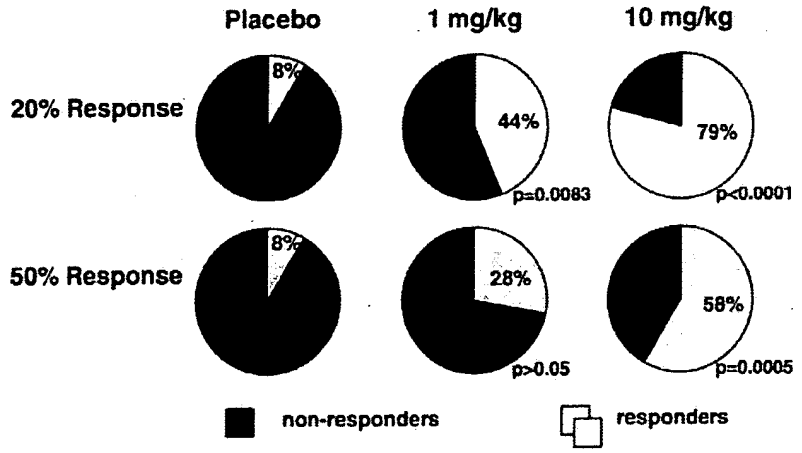

Figure 4. Responses at week 4 in patients treated with high (10 mg/kg) or low (1 mg/kg) dose cA2 or placebo. Patients received a single infusion of cA2 or placebo at entry (week 0). Responses were calculated according to Paulus 20% or 50% criteria, as described in the text. p values represent significance of changes compared with placebo by Fisher's exact test.

and observers' assessments of disease severity all showed highly significant improvements. These changes were matched by improvements in the ESR and CRP, particularly in the high-dose cA2 group. As in the open label study, the infusions were well tolerated and though a variety of adverse events were seen, the overall safety profile of cA2 was good, and the adverse events were not greater at high doses of cA2 than placebo.

One element of particular interest in this study was the improvement in the hemoglobin concentration in patients treated with high-dose cA2, compared with a fall in placebo-treated patients. Although these changes were small in magnitude, they occurred over a period of only 4 weeks in the setting of significant venesection for safety monitoring, and the difference between the changes in placebo and high-dose cA2 groups was highly significant. The anemia of chronic disease seen in RA is multifactorial in origin but recent evidence suggests that TNFα has a direct effect in the bone marrow in suppressing erythropoiesis (Roodman 1987, Johnson et al. 1989). While the improvements in hemoglobin in cA2 treated patients might be simply a reflection of improvement of overall disease state, a more interesting hypothesis is that cA2 is directly interfering with TNFα mediated suppression of erythropoiesis in the bone marrow.

The results of this study confirm that cA2 is a highly effective agent in the short-term suppression of RA and strongly support the hypothesis that TNF is a significant contributor to the inflammtory state in this disease. The findings do not however directly address the question of the relative importance of TNFα and IL-1 in disease pathogenesis. No full-length reports are yet available describing the use of specific IL-1 blockers in RA, but preliminary data presented in abstract form suggest some therapeutic efficacy for both IL-1ra (Lebsack et al. 1993) and a soluble receptor for IL-1 (Drevlow et al. 1993).

The data presented so far suggests that cA2 may find a role as a remission inducing agent in RA, or in the control of acute disease flares. A more ambitious goal for a new therapeutic agent, however, would be long-term disease suppression and in particular the achievement of disease modification. Although many of the currently available drugs in the treatment of rheumatic disease are termed 'disease modifying', the evidence that they do in fact alter outcomes is at best arguable (Brooks 1993). TNFα has major effects on cartilage metabolism, including the inhibition of collagen synthesis and the stimulation of collagenase production by fibroblasts and synovial cells, together with both direct and indirect effects on proteoglycan metabolism (reviewed by Vassalli 1992). It might be expected, therefore, that long-term TNF blockade would favorably influence cartilage metabolism and reduce tissue damage.

Experience with cA2 suggests that, although it is a reliable disease suppressing agent, the responses are transient, with patients showing disease relapse after a period of months after the antibody disappears from the circulation. In our first experience in the repeated use of cA2, we administered cycles of therapy to a small

number of patients originally enrolled in the open label trial. Seven patients completed between 2 and 4 complete treatment cycles, with each cycle administered upon evidence of disease relapse. The findings have been reported in full recently (Elliott et al. 1994b), with repeated responses after each cycle administered, and maintenance of response magnitude, but a trend (non-significant) to shortening response duration. Of particular interest in this study was the tissue of immunogenicity, since the murine variable region of cA2 might be expected to stimulate anti-murine responses upon repeated dosing. Although only 1 patient had developed an anti-murine response in the original open label trial (unpublished data), 50% of the patients re-treated with cA2 developed such responses, mostly at a low titer.

The clinical significance of these immunological responses to injected antibody are as yet unclear, but it is possible that they could limit therapeutic efficacy with long-term treatment either through accelerated clearance of injected cA2, or by blocking cA2 binding sites to TNFα. Overcoming these responses is likely to represent the major therapeutic challenge for the repeated use of cA2, or indeed any other engineered thereapeutic antibodies, since even 'humanized' antibodies are potentially immunogenic (Isaacs et al. 1992). While a number of approaches could be adopted to circumvent the development of such responses, administration of antibodies to CD4 to block the development of antiglobulin responses to concurrently administered anti-TNF antibody has been discussed in the previous section on animal models.

Preliminary reports from 2 other studies targeting TNFα in RA have been made recently. In one, a humanized murine antibody (CDP571) was administered to patients with active rheumatoid arthritis in a placebo controlled, dose escalation study (Rankin et al. 1994). The antibody was well tolerated and significant improvements were seen in a number of individual disease activity measures, including the tender joint count, the pain score and the ESR. A second reported study employed a fusion protein, comprising recombinant human p75 TNF receptor coupled to a human IgG1 Fc framework (Moreland et al. 1994). This dose-ranging study incorporated intravenous loading at entry, with twice-weekly subcutaneous administration of the fusion protein for maintenance therapy. Significant improvements were seen compared to baseline for the tender and swollen joint counts, the patient and physician global assessments and the duration of morning stiffness, together with the ESR. These data lend support to our findings and further validate the clinical efficacy of TNFα blockade. The current status of trials in progress with anti-cytokines mostly published in abstract form, is summarized in Table IV.

MECHANISM OF ACTION OF ANTI-TNFα ANTIBODY THERAPY

The 'pleiotropy' of TNF action, jargon for its multitude of effects on various cell types, driving multiple biological processes, is the likely reason for the marked

TABLE IV
Anti-cytokines in clinical trials in RA

Target	Therapy	Results
TNFα	● Monoclonal anti-TNFα antibody:	
	– Chimeric (cA2) (Centocor)+	*RCT: beneficial
	– humanised (CDP571) (CellTech)	Dose escalation: beneficial
	● Soluble TNF-R-Ig fusion proteins:	
	– TNFR:Fc (p55) (Roche)	In trials: beneficial
	– TNFR:Fc (p75) (Immunex)	Dose ranging: beneficial
IL-1	● IL-1RA (Synergen)	Dose ranging: beneficial
	● Soluble IL-1R (Immunex)	RCT: trend to beneficial
IL-6	● Monoclonal anti-IL-6 antibody	
	– murine (BE-8)	Open label: beneficial

*RCT=randomized placebo-controlled trial.
+=published as full papers, rest are published in abstract form.

effects of anti-TNF therapy *in vivo*. However, it remains to be established which of these many effects of TNF, inhibited by anti-TNF, are of central importance in the therapeutic effect. Our current working hypothesis, based on the results so far that the cA2 anitbody has two major effects. It interrupts the cytokine cascade *in vivo*, just as it does in the *in vitro* rheumatoid synovial cultures. The best clinical evidence for interruption of the cytokine cascade *in vivo* is the rapid and marked reduction in the CRP, the rise of which in RA reflects the cytokine activation of the hepatocytes, probably chiefly by IL-6, but possibly involving any of the cytokines whose signals are mediated by gp130 (e.g. LIF, IL-11). Supporting evidence for widespread cytokine blockade has been obtained by the fall in serum IL-6 levels by ELISA (Fig. 3). Cytokine blockade and diminution of events downstream is probably of major importance in the rapidity of onset of clinical benefit, and in the reduction in local inflammatory features, such as morning stiffness, pain and joint swelling.

However, an important feature of cA2 therapy is the duration of clinical benefit, much beyond the fall of serum levels of anti-TNF to levels not capable of neutralizing TNFα bioactivity within the RA joints. A number of lines of evidence have converged to suggest the hypothesis that cA2 has a major effect on the recruitment and trafficking of blood cells to the joint. Endothelium in RA synovium has been reported to express increased levels of adhesion molecules for leukocytes, such as E-selectin, VCAM-1 and ICAM-1 (Koch et al. 1991, Fischer et al. 1993, Johnson et al. 1993, Cronstein 1994, Szekanecz et al. 1994). Since blood cells, neutrophils, but especially macrophages and T cells, are needed for maintaining the disease process and do not proliferate much locally, interruption of the influx of cells may be of major significance in the duration and depth of

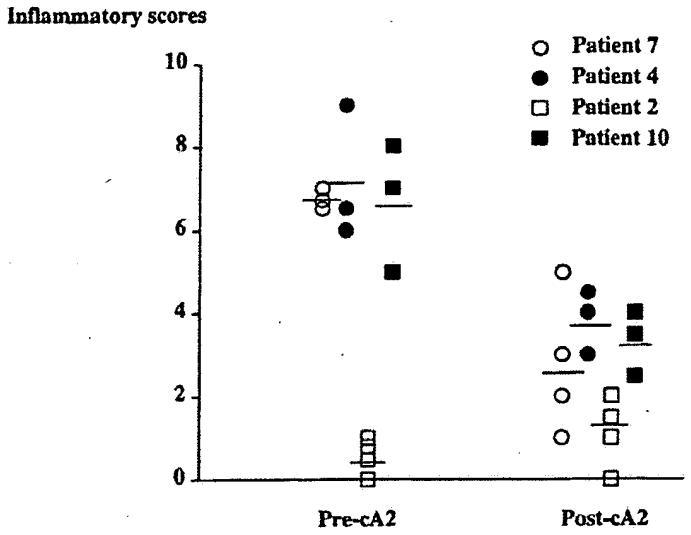

Figure 5. Reduction of inflammation in the synovial membrane in RA patients treated with anti-TNFα monoclonal antibody (cA2). Multiple synovial biopsies were taken before and 14 days after cA2 infusion from 4 patients in the 'open-label' clinical trial and analyzed for the degree of inflammation. Two distant sections from each biopsied sample were graded by scoring the thickness of the lining layer, cellular infiltration and the number of lymphoid aggregates (Firestein et al. 1991). Each point in the figure represents the average value of the two sections of each biopsy.

benefit. Moreover, it suggests that blocking TNF long-term could interrupt the destructive process itself, which needs cell influx to sustain it, rather than merely reducing the clinical features such as pain and swelling more effectively than existing drugs.

The clues we have obtained to support the role of trafficking have come from synovial histology, where the adhesion molecules on synovial endothelium have also been studied, and from analysis of the serum marker of endothelial activation, soluble E-selectin.

Synovial biopsies before and 14 days after cA2 infusion were available from 4 patients in our first 'open-label' clinical trial. In each patient, at least 3 biopsies were taken from the same knee joint before and after therapy. Two distant sections from each block of biopsied tissue were stained and examined for thickness of the lining layer, cellular infiltration and the number of lymphoid aggregates. These parameters were used to score the degree of inflammation of the synovial membrane. We found the score to be high in 3 patients and only minimally raised in 1. In the 3 patients with the highest inflammatory score, there was a reduction from a mean value of 6.8 to 3.5 following cA2 therapy (Fig. 5). Other histological

features were assessed. The synovial lining layer is relatively uniform in individual joints, in contrast to the sublining layer which shows regional variability. The lining layer decreased from 6–8 cells thick to 3–4 cells thick following therapy. Lymphoid aggregates are patchy in distribution; nevertheless these were scored and showed a reduction from 5 to 3.5 per low-power field. Since neovascularization is a prominent feature of the RA synovium, the number of blood vessels (defined by staining with antibody to von Willebrand Factor and a specific anti-endothelial antibody EN4) (Ruiter et al. 1989) was compared. There was no significant change in the number of vessels in pre- and post-treatment specimens.

Since trafficking of leukocytes to the joint is an important process in the pathogenesis of rheumatoid arthritis and augmented expression of endothelial adhesion molecules is involved in this process, we monitored the expression of ICAM-1, VCAM-1 and E-selectin on vascular endothelium in the biopsies. ICAM-1 and VCAM-1 were detected on most vascular endothelial cells, as well as many of the lining layer cells. This is expected, based on the expression of these molecules on antigen-presenting cells as well as endothelial cells. There was a tendency for VCAM-1 staining on endothelial cells, but not on lining cells, to be reduced post-treatment, but no changes in ICAM-1 expression on endothelium were noted. Antibody to E-selectin exclusively stained 3–28% of vascular endothelial cells on capillaries and venules. The expression of E-selectin was significantly reduced in the post-treatment samples (Fig. 6).

Recently it has become apparent that many adhesion molecules, like cytokine receptors, also exist in the serum in a soluble form, to complement the cell surface form. Their function in the serum is not clearly documented, and unlike sTNF-R, which was discovered as a urinary TNF inhibitor, it is not yet known whether they are competitive inhibitors. However, it is known that these molecules are derived from proteolytic cleavage of the surface form, as no alternatively spliced mRNA has been found, at least for ICAM-1 and E-selectin (Gearing & Newman 1993). Release of soluble ICAM-1 and E-selectin has been found to correlate with expression of these molecules on the surface of cytokine-activated cultured endothelial cells (Leeuwenberg et al. 1992, Pigott et al. 1992). Elevated serum levels of ICAM-1, VCAM-1 and E-selectin have been observed in RA (Cush et al. 1993, Koch et al. 1993, Wellicome et al. 1993), although only ICAM-1 and VCAM-1 appear to correlate with disease severity (ESR) (Aoki et al. 1993, Mason et al. 1993).

In order to further assess the significance of the reduction in E-selectin observed on synovial blood vessels, levels of soluble E-selectin were assayed in serial serum samples, and were found to be diminished by cA2 therapy in 19/20 patients. The earliest observed decrease was at day 7, although serum levels appeared to be attenuated even at 4 and 6 weeks (Fig. 7). In some patients levels returned to baseline by day 56 (in 5/20 levels were rising by weeks 6 to 8); however, in most patients levels were still below pre-treatment levels at that time. The initial levels

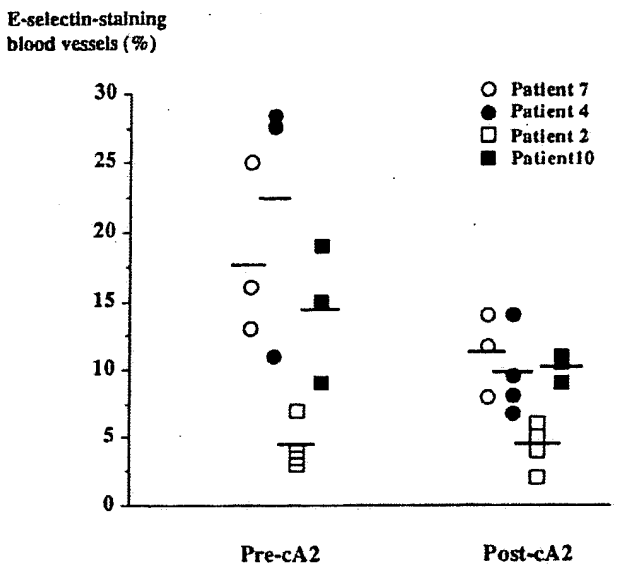

Figure 6. Decrease of E-selectin expression on endothelial cells in the synovial membrane in RA patients treated with anti-TNFα monoclonal antibody (cA2). The synovial biopsies (as stated in Fig. 1) were stained with a mouse monoclonal anti-human E-selectin antibody (BBA1). The expression of E-selectin on endothelial cells was reduced in the post-treatment samples in the same 3 patients that showed reduced inflammatory scores.

in the 20 patients varied considerably from 41–224 ng/ml (mean normal 47.6 ng/ml). In 1 patient, in whom no diminution of soluble E-selectin levels was observed, the initial levels were in the normal range (<50ng/ml).

Levels of soluble ICAM-1 were assayed in only 3 patients, and also exhibited an apparent decrease following cA2 therapy. Mean serum ICAM-1 levels decreased from 351.7 ng/ml (pre-infusion) to 262.3 ng/ml by day 7, although this difference was not statistically significant due to the low number of patients tested. Four weeks post-infusion the levels of ICAM-1 appeared to be returning to pre-infusion values. Finally, sera from a total of 10 patients were assayed for soluble VCAM-1. There was no significant difference detectable in levels of soluble VCAM-1 at any time after cA2 infusion (Fig. 7).

SUMMARY

Rheumatoid arthritis is a common cause of chronic disability for which current therapies are of limited value in controlling the disease process and outcome. Our initial approcah to understanding the pathogenesis of RA and defining a novel

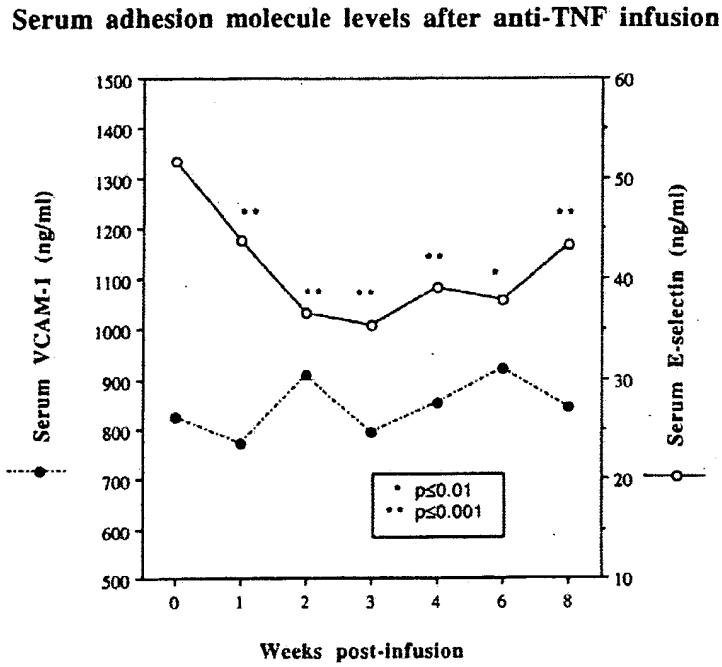

Figure 7. Serum adhesion molecules after anti-TNF infusion. Circulating levels of serum E-selectin (-o-) VCAM −1 (-●-) were measured by ELISA (British Biotechnology Products Ltd, UK) after infusion of cA2. Values are means of 20 and 10 patients respectively. Significant differences versus pre-infusion values were determined by Wilcoxon signed rank test (*) $p<0.01$, (**) $p<0.001$.

therapeutic target was to investigate the role of cytokines by blocking their action with antibodies on cultured synovial-derived mononuclear cells *in vitro*. These investigations suggested that neutralization of TNFα with antibodies significantly inhibited the generation of other pro-inflammatory cytokines also over-produced, such as, IL-1, GM-CSF, IL-6 and IL-8. The implication that blockade of a single cytokine, TNFα might have far-reaching effects on multiple cytokines and thereby exert significant anti-inflammatory and protective effects on cartilage and bone of joints, was tested in arthritic DBA/1 mice immunized with collagen II. Impressive amelioration of joint swelling and joint erosions in this model encouraged clinical trials with a monoclonal anti-TNFα antibody. The cA2 chimeric anti-TNFα high-affinity antibody was initially tested in an open-label study at a dose of 20 mg/kg on 20 patients, with substantial and universal benefit. Subsequently, a randomized placebo-controlled double-blind trial was performed on 73 patients comparing a

single intravenous injection of placebo (0.1% human serum albumin) with two doses of cA2. Using a composite disease activity index, at 4 weeks post infusion, 8% of patients receiving placebo improved compared with 44% receiving 1mg/kg cA/2 and 79% receiving 10 mg/kg. Between 2 to 4 repeated cycles of cA2 were administered to 7 patients and all patients showed improvement of a similar magnitude with each cycle. These data support our proposition that TNFα is implicated in the pathogenesis of RA, and is thus a key therapeutic target. Monoclonal anti-TNFα antibodies control disease flares and are candidate agents for longer-term control of RA, although repeated therapy with cA2 is associated with anti-idiotypic responses in 50% of patients and a trend toward shortening of the duration of response. In the DBA/1 arthritic mice, synergy of action of anti-TNF and anti-CD4 is observed together with suppression of an anti-globulin response, indicating one way in which benefit might be augmented in the future.

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[Monoclonal antibody induces apoptosis against cancer cells]

[Article in Japanese]

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ErbB-2, a member of the epidermal growth factor(EGF) receptor tyrosine kinase family, is often overexpressed and/or amplified in breast, ovarian and gastric cancers, and other malignancies. ErbB-2 is a candidate as one of the best target molecules for cancer therapy. Many anti-ErbB-2 monoclonal antibodies(MoAbs) have been developed. An inhibitory humanized MoAb shows clinical responses in some breast cancer patients, both with MoAb alone and in combination with Cisplatinum or other anti-cancer drugs. A mouse-human chimeric anti-ErbB-2 MoAb CH401 was established and characterized in our laboratory. CH401 is able to kill cancer cells overexpressing ErbB-2 both in vitro and in vivo. The analysis of this tumor growth inhibition by CH401 made it clear that the cytotoxicity was induced by apoptosis. These results may suggest that CH401 has a therapeutic potential for ErbB-2 overexpressing cancers. This approach may be particularly valuable as a new type of cancer therapy.

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Bispecific Monoclonal Antibody Therapy of B-Cell Malignancy

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Bispecific monoclonal antibodies (bsAbs) that recognize CD3 with one arm and a tumor associated antigen with the other arm can retarget T-cells toward tumor cells in an MHC independent manner, thereby combining the specificity of monoclonal antibodies with the power of the cellular immune system. B-cell malignancies are particularly attractive as targets for anti-CD3-based bsAb therapy because of their sensitivity to other forms of antibody therapy, and the extent to which B-cells and T-cells communicate at the molecular level. BsAbs that recognize CD3 and a number of antigens on malignant B-cells have been shown in vitro to be capable of retargeting T-cells. In animal models of B-cell malignancy, bsAb can eliminate tumor loads that are resistant to unmodified monoclonal antibody therapy. Ongoing early clinical trials in advanced B-cell lymphoma indicate CD3-based bsAbs have significant biologic effects, and suggest they have anti-tumor activity as well. A number of significant questions relating to bsAb therapy of B-cell malignancies remain. It is unclear what role both endogenously produced and exogenously administered cytokines are likely to play. Further exploration of whether bsAb can induce T-cells to target to tumor will also be required before the true promise of this novel form of immunotherapy can be determined.

KEY WORDS: Bispecific monoclonal antibodies therapy B-cell malignancy

Therapy with monoclonal antibodies (mAbs) has shown promise in the area of B-cell malignancies. Significant tumor regressions have been noted after treatment with B-cell-specific radiolabeled mAbs,[1,2] immunotoxins[3] and unlabeled mAbs.[4] However, each of these modalities has its limitations. Non-specific toxicity from radioimmunotherapy can result in immunosuppression and myelosuppression, particularly if the marrow is involved with tumor. Immunotoxins often cause liver or vascular injury and tend to be immunogenic, thus repeated use is often not possible. Unlabeled mAbs are well tolerated and can localize in tumor, but in most cases do not activate immune effector mechanisms sufficiently to induce tumor shrinkage. Indirect evidence from a number of areas indicates T-cell immunity plays a key role in the control of abnormal B-cell proliferation. Patients with compromised T-cell function due to HIV infection or the use of immunosuppressants develop B-cell neoplasia at a high rate. Relapse following bone marrow transplantation of patients with acute lymphoblastic leukemia is higher if no acute or chronic graft-versus-host disease occurs.[5] Therapy with donor peripheral blood lymphocytes, most of which are T-cells, has been used to reverse post-transplant EBV-induced lymphoproliferation.[6]

An immunotherapy that combines the specificity of mAbs with the potency of T-cell-based immunity might be more specific and less toxic than either form of therapy alone. One such approach is the use of bispecific antibodies (bsAbs) which simultaneously bind to immune effector cells and tumor cells via a tumor associated antigen.[7] A number of different mAbs have been used for the effector cell-specific arm of bsAbs,

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including those that recognize a variety of Fc receptors.[8-12] CD3 is attractive as the antigen recognized by the effector specific arm of bsAbs for a number of reasons. By definition, CD3 is present on all T-cells. CD3 also plays a key role in the transduction of signals involved in T-cell activation, as indicated by the T-cell activation and proliferation that results when T-cells are treated with anti-CD3.[13] Anti-CD3 cross-links CD3 and induces T-cell activation most efficiently when the antibody is anchored on another cell, such as occurs when FcR-bearing cells are present. BsAb composed of an anti-CD3 arm and an anti-tumor arm can also cross-link CD3 and induce T-cell activation. This effect is more pronounced when cells bearing the target antigen are present (Figure 1). BsAb, in the presence of T-cells and tumor cells also induces the release of cytokines which may enhance T-cell activation locally and inhibit the growth of bystander tumor cells.[14] Further, each T-cell is capable of killing more than one tumor cell if the T-cell is recoated with bsAb.[15]

BsAbs can be produced by a variety of techniques. Chemical heteroconjugates can be created by the chemical linking of either intact antibodies or antibody fragments of different specificities.[10,16] While production of bispecific heteroconjugates from intact IgG is relatively straight forward, the resulting complexes are at least twice the size of the original mAbs which could limit their ability to penetrate into tumors. In addition, producing large quantities of chemical heteroconjugates in a reproducible manner is difficult. Glennie has described a technique involving disulfide exchange using $F(ab')_2$ fragments that can overcome some of the problems associated with the heterogeneity of chemical heteroconjugates.[17] This technique requires that both antibodies be susceptible to enzymatic cleavage, and that rigorous purification be used to separate parental antibodies from bsAb because of the tendency for homodimers to form. Kostelny *et al.* have produced a high yield of bsAb in a reproducible manner by using genetic techniques.[18] This approach involves the creation of $F(ab')_2$ connected via a shortened Fc to the leucine zipper region of the transcription factors Fos and Jun. The preferential heterodimer formation displayed by the Fos and Jun leucine zipper peptide sequences results in a high yield of highly pure bsAb that lacks functional Fc. Other genetic techniques have also been used to produce bsAbs.[19,20]

Hybrid-hybridomas (also known as quadromas) that contain two productive immunoglobulin heavy chain and two productive light chain allels, can also be used to produce bsAb.[21] Such cells are created by fusing two hybridoma cell lines together. Hybrid-hybridomas secrete individual IgG molecules which are monovalent for each of the two distinct antigens recognized by the parent antibodies. Structurally, bsAbs produced in this manner are intact IgG, and are monovalent for two distinct antigens. However, hybrid-hybridomas produce both bsAb (in the form of bispecific IgG) and antibodies that are monospecific (and bivalent) for each of the two antigens recognized by the parent hybridomas. Further, light chain/heavy chain fidelity does not always occur as was demonstrated in Kohler and Milstein's original description of the hybridoma technique.[22] In all, there are 10 possible heavy and light chain combinations that could be produced by a hybrid-hybridoma cell line. Only one of these is the desired bsAb. Heavy chain–light chain pairing in hybrid-hybridomas appears to vary from cell line to cell line, with some exhibiting preferential pairing of chains from each parental antibody (heavy chain and light chain from each parental hybridoma tending to pair together), while others demonstrate no such preference (heavy and light chain pairing is random).[23] Some degree of purification of the bispecific component is therefore necessary prior to the use of such bsAbs.

Anti-CD3/anti-tumor bsAbs in the form of both heteroconjugates and bispecific IgG from hybrid-hybridomas have been evaluated in a number of in vitro systems. BsAb has been shown to direct T-cell mediated lysis of renal cell carcinoma,[24,25] melanoma,[13,26,27] glioma,[28] ovarian carcinoma[29,30] and colorectal carcinoma.[31] In xenograft animal models, bsAb has been shown to be significantly superior to monospecific mAb at preventing tumor growth.[27,32-34] Limited clinical trials suggest bsAb can enhance the antitumor effect of regionally administered T cells in the local treatment of glioma,[35] and treatment of peritoneal carcinomatosis with ovarian[36] and colorectal carcinoma.[37] B-cell malignancies are particularly attractive as targets for anti-CD3-based bsAb therapy. As outlined above, B-cell malignancies are the most sensitive tumors to other forms of mAb-based therapy. T-cell infiltration into malignant nodes is common. Immunophenotyping of B-cells is extensive, and supplies a variety of target antigens for the tumor-specific arm of the bsAb. Perhaps most importantly, T-cells communicate extensively with B-cells via a variety of molecules including CD28, CD4, CD8 and gp39 on the T-cell and HLA class II, the B7 family, and CD40 on the B-cell. These interactions normally play key roles in control of B-cell proliferation and T-cell ac-

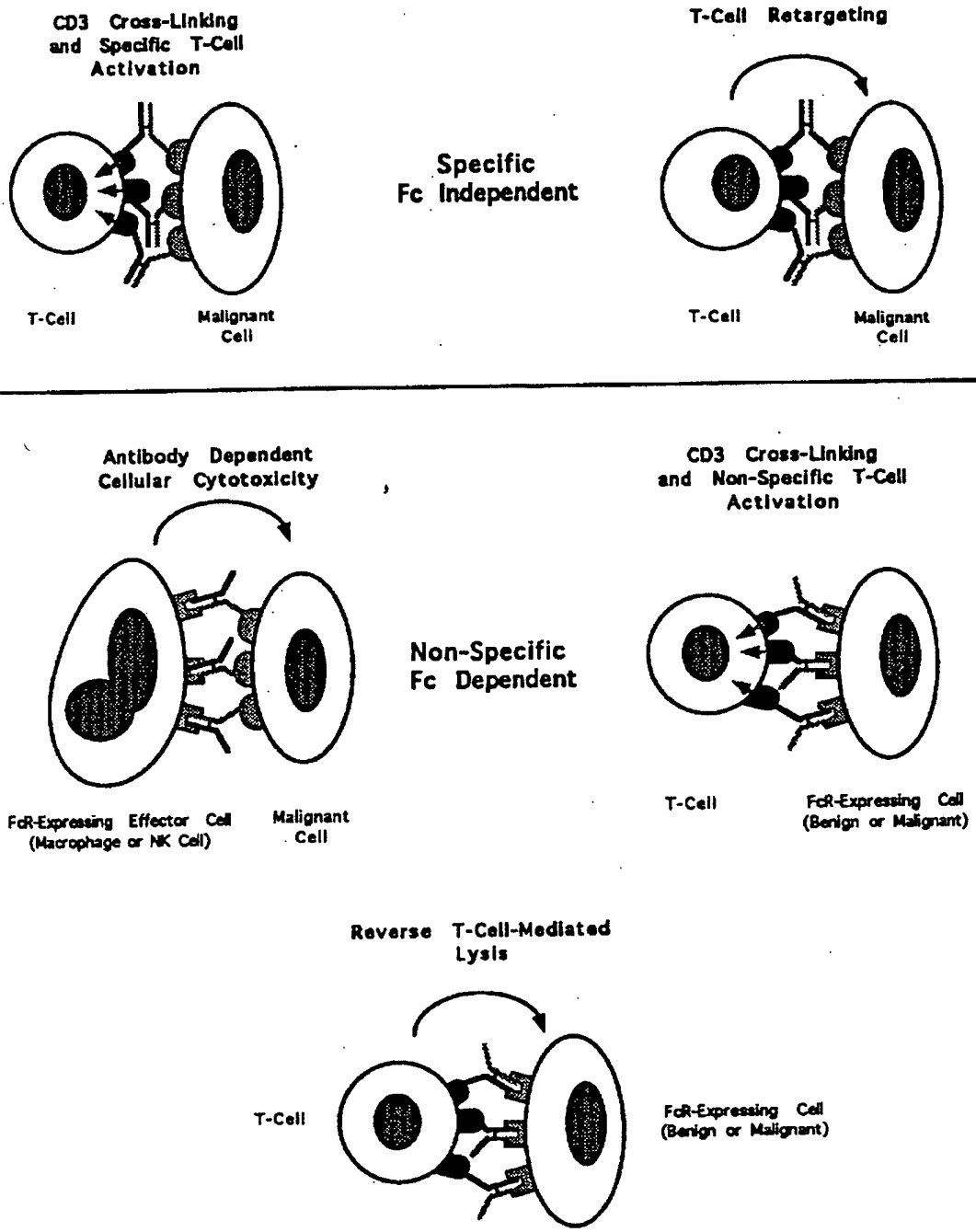

Figure 1 Specific and non-specific effects of bsAbs: Anti-CD3-based bsAbs can have a number of different effects based on binding via the CD3 specific arm, tumor specific arm, or Fc. Specific effects related to the CD3 and tumor specific arms would be mediated by $F(ab')_2$ as well as monospecific antibody, and would include T-cell activation induced by CD3 cross-linking, and retargeting of the T-cell. Non-specific effects could result from the presence of a functional Fc. Such effects could include standard antibody dependent cellular cytotoxicity (mediated by the tumor specific arm and Fc), non-specific activation of the T-cell (mediated by the CD3-specific arm and Fc), and CD3-mediated reverse lysis (mediated by the CD3-specific arm and Fc).

tivation, and contribute to lysis of target cells by T-cells[38].

For bsAb to be effective in the treatment of B-cell neoplasia, systemic therapy is required. Such an approach is more complex than that seen with regional bsAb therapy. BsAb must not only retarget T-cells, but must reach the site of the malignant cells. In addition; cytotoxic lymphocytes need to be present (Figure 2). Studies of bsAb therapy in animal models of B-cell malignancy indicate this can occur. Demanet *et al.*[39] produced a bispecific IgG using the hybrid-hybridoma technique. This bsAb recognized CD3 and the idiotype expressed by the 38C13 murine B-cell lymphoma, and was able to inhibit tumor growth in syngeneic, immunocompetent mice. Inhibition of tumor growth was also seen in a strain of C3H mice that have a profound defect in macrophage differentiation and markedly decreased FcR-γ-mediated antibody dependent cellular cytotoxicity (ADCC), further supporting the contention that T-cell retargeting by bsAb, and not ADCC, was responsible for the antitumor effect. Similar findings were noted by the same group in the BCL_1 lymphoma model.[40] Weiner and Hillstrom also studied anti-idiotype x anti-CD3 therapy in the 38C13 model.[41] Their studies confirmed the results of Demanet *et al.*, and evaluated the specificity of anti-idiotype x anti-CD3 therapy. Mice were inoculated with antigen-positive tumor cells that were intentionally mixed with a small number (0.5%) of target antigen-negative idiotype variant tumor cells. The antigen-negative cells emerged in the tumors that developed despite bsAb therapy. Thus, no inhibition of bystander cells was seen. These studies point to the specificity of bsAb therapy, and the importance of selecting a target antigen that is uniformly expressed on the malignant cells.

More recently, Weiner *et al.* have evaluated the importance of bsAb Fc, and the role it plays in T-cell activation. This was done by comparing 2 bsAb preparations in the 38C13 murine B-cell lymphoma model.[42] One preparation used in these experiments consisted of bispecific IgG, and was obtained from a hybrid-hybridoma. This bispecific IgG was an intact IgG, and therefore contained functional, if hybrid, Fc. The second preparation was a modified bispecific $F(ab')_2$ produced genetically using the leucine zipper technique described by Kostelny *et al.*[18] This preparation lacked any detectable Fc function. In vitro analysis demonstrated both bispecific IgG and bispecific $F(ab')_2$ induced tumor cell lysis by pre-activated T-cells. However, bispecific IgG induced T-cell proliferation in the absence of tumor cells while bispecific $F(ab')_2$ did not. Thus, both preparations were able to retarget T-cell mediated lysis, whereas the bispecific IgG was also effective at inducing non-specific T-cell activation. Most likely this was due to cross-linking of CD3 by bsAb Fc binding to Fc-receptors (Figure 1). In vivo results were consistent with these findings. A single dose of bispecific IgG inhibited tumor growth, while a single dose of bispecific $F(ab')_2$ had minimal antitumor effect. Bispecific $F(ab')_2$ was capable of preventing tumor growth and improving survival when mice were also treated with T-cell activators (IL-2 or staphylococcal enterotoxin B), or given repeated bispecific $F(ab')_2$ doses. The therapeutic response to bsAb therefore was not dependent on functional Fc mediating ADCC, however T-cell activation induced by either Fc-mediated cross-linking of CD3 or the addition of other T-cell activators was required. These studies demonstrate that the use of bifunctional constructs that lack functional Fc may allow for separate manipulation of T-cell retargeting and T-cell activation.

A number of molecular structures found on human B-cell malignancies have been explored as potential tumor targets for anti-CD3-based bsAb therapy. Each has its own advantages and disadvantages. The idiotype is tumor specific, and has served well in the animal models, however production of tailor-made bsAbs for each patient's malignancy would be required. Further, the emergence of idiotype variants is likely to be a problem as it has been in clinical trials of monospecific anti-idiotype therapy.[43] Nevertheless, anti-CD3 × anti-idiotype bsAbs can induce lysis of human lymphoma cells by human T-cells.[44] CD3 × CD10 bsAb have been shown to be capable of inducing lysis of CD10(+) ALL cells.[45] However, targeting towards large numbers of benign cells would be expected when molecules that are expressed by both B-cells and non-B-cells, such as CD10, CD5 or HLA class II, are targeted. The antibody designated 1D10 is promising, as it appears to recognize a glycosylation variant of HLA-DR that is almost exclusively expressed by malignant B-cells.[46] Link and Weiner demonstrated that an anti-CD3 × 1D10 bsAb can induce T-cells to lyse a variety of allogenic and autologous malignant B-cells.[47] 1D10 has not yet been evaluated clinically, thus its true pattern of reactivity and potential are as yet unknown. CD19 and CD20, which are lineage specific and are expressed strongly by both normal and neoplastic B-cells, are attractive as targets. Benign B-cells are quickly replenished from populations of cells that do not express these antigens, thus clearance of benign B-cells is unlikely to result in signif-

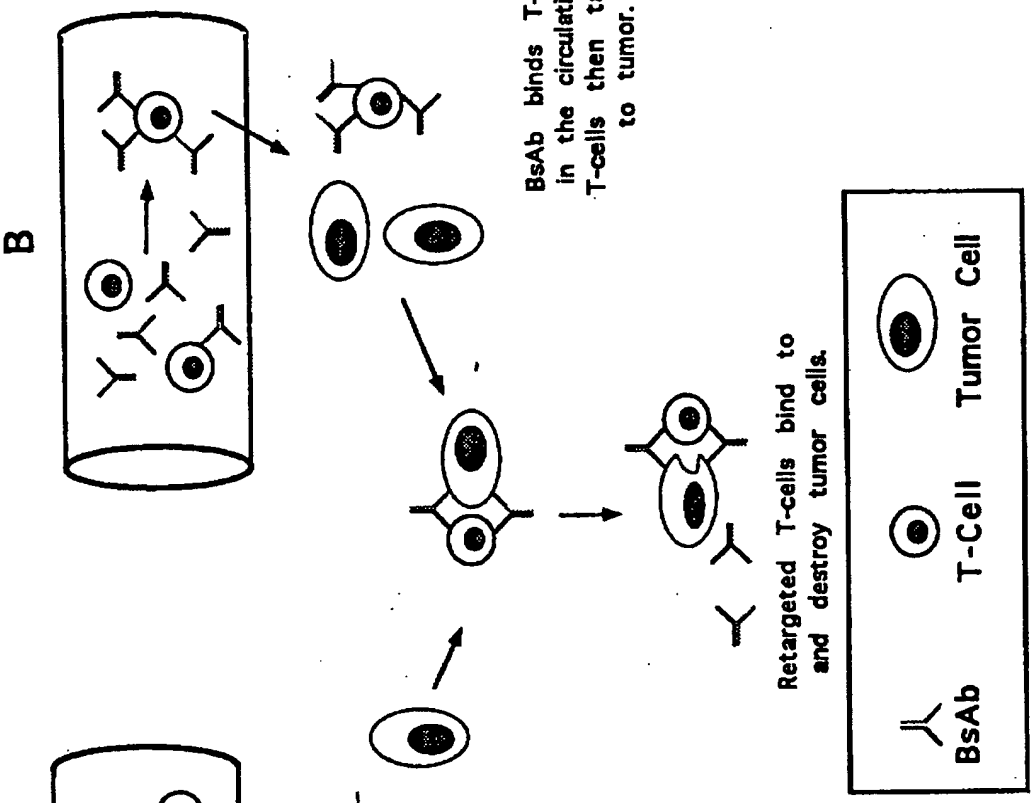

Figure 2 Steps which need to take place for systemically administered bsAb to induce lysis of malignant cells: Systemically administered bsAb must reach the site of the tumor and bind to both tumor cells and T-cells. This may result from bsAb exiting the circulation and binding to T-cells that are already in the tumor, or bsAb binding to T-cells in the circulation, and inducing them to target to tumor. (A) BsAb exits the circulation in an unbound state. In the tumor, bsAb binds to both malignant cells and in situ T-cells. When bsAb links the T-cell to the tumor cell, it induces T-cell activation by cross-linking CD3. Tumor cells are then destroyed by the retargeted and activated T-cells. (B) BsAb binds to T-cells within the circulation. The T-cells coated with bsAb target to tumor. The bsAb then binds to the tumor cell. This induces T-cell activation by cross-linking CD3, and results in tumor cell destruction.

icant toxicity. Several groups have evaluated CD3 × CD19 bsAbs obtained from either hybrid-hybridomas or chemical conjugation. Such bsAbs can induce lysis of more differentiated malignant cells (CLL) as well as those that are less differentiated (ALL).[48,49] Bohlen et al demonstrated co-stimulation of T-cells via CD28 enhances T-cell activation and lysis mediated by CD3 × CD19.[50] Further, bispecific anti CD28 × CD22 enhanced lysis mediated by CD3 × CD19.[51] These results point to the importance of T-cell activation when using anti-CD3-based bsAb.

For the reasons outlined above, CD19 was selected as the target antigen for a clinical trial of bsAb therapy. This ongoing trial by De Gast and coworkers involves the evaluation of an anti-CD3 × CD19 bsAb produced by M. Clark using the hybrid-hybridoma technique. The hybrid-hybridoma was created by the fusion of YTH12.5, a rat hybridoma that secretes an IgG2b anti-CD3, with MG1CD19, a mouse hybridoma that secretes an IgG1 anti-CD19.[52,53] This bsAb has the advantage of being composed of a rat IgG2b-lambda and a mouse IgG1-kappa, thus every chain can be separately identified and overexpression of one of the chains can be easily detected.

CD3 × CD19 was first extensively tested in vitro to evaluate its capacity to induce cytotoxicity. Both TCR$\alpha\beta$ and TCR$\gamma\delta$ T cell clones were able to mediate lysis of CD19-expressing target cell lines in the presence CD3 × CD19.[49] Freshly isolated tumor cells were killed as well. Minimal lysis was seen in the absence of bsAb. CD19(−) cell lines were not susceptible to bsAb mediated cytotoxicity. Lysis of CD19(+), CD32(−) cell lines was also seen, as was lysis after the addition of anti-CD32, further suggesting that reactivity with CD19, and not FcR, was responsible for target cell destruction. Peripheral blood T-cells of normal donors, activated in vitro by PHA or CD3 mAb combined with IL-2, were also able to kill freshly isolated CD19+ tumor cells. BsAb was also capable of inducing lysis of malignant cells by autologous activated T-cells.[54]

The aim of this Phase I trial of intravenously administered BsAb was to investigate the toxicity of intravenously administered CD3 × CD19 bsAb in patients with end stage CD19+ leukemia/lymphoma. The three patients evaluable to date include 2 with low grade NHL and 1 with intermediate grade NHL. All were chemotherapy resistant, 15–70 years of age, had a WHO performance grade 0, 1 or 2 and supplied informed consent. Patients with severe cardiac, pulmonary, neurologic or metabolic disease, persistent organ failure, or a positive test for HIV were excluded BsAb was administered as follows:

day 1	0.1 μg bsAb intracutaneous, 2 hrs later 10 μg bsAb I.V. over 10 min.
day 2	100 μg bsAb I.V. over 15 min.
days 3, 4	400 μg bsAb I.V. over 30 min.
days 5, 6	1000 μg bsAb I.V. over 30 min.
days 7, 8	2500 μg bsAb I.V. over 30 min.
day 9	5000 μg bsAb I.V. over 30 min.

The major toxicity noted was fever and chills. Interestingly, this was noted one hour after bsAb infusion in the patient with circulating malignant cells, but was not seen until 3–4 hours after bsAb administration in the two patients with enlarged lymph nodes but no detectable circulating cells. This suggests that fever and chills may be due to bsAb-induced cytokine release that occurs when bsAb binds to tumor cells and cross-links CD3 on T-cells. Minor toxicities (grade I WHO scale) included thrombocytopenia (3 patients), mild hypotension (1 patient), increase in bilirubin (1 patient), erythema with eosinophilia (1 patient) and transient minor decrease of Ig levels (3 patients). No other organ toxicity was found. No granulocytopenia or monocytopenia was observed. In addition, no detectable human antibodies to mouse Ig (HAMA) or rat Ig (HARA) were found. Transient severe T and B lymphocytopenia was noted, and resolved gradually after the bsAb infusions were stopped.

Although this was a phase I study, some evidence for efficacy was noted in the two patients with low grade NHL. Both experienced a decrease in lymph edema caused by grossly enlarged lymph nodes. One of these patients also had a decrease in splenomegaly. Progression free survival of 6 months was noted in the one patient, who eventually died of a cerebral hemorrhage. The other patient has had no progression during 2 months follow-up. The patient with intermediate grade lymphoma had progression during bsAb therapy and died 3 months later from progressive lymphoma. Due to the small number of individuals studied to date, it is difficult to speculate on the significance of the findings thus far. Symptoms including fever and chills were most likely secondary to bsAb-induced cytokine release from T-cells which were activated when they came in contact with tumor cells, although it is also possible that some of the cytokine release came from T-cells that were activated when bsAb bound to T-cells and benign B-cells, via either CD19 or FcR.

Animal studies suggest cytokines can contribute to

the anti-tumor effects of bsAb, however, the role of both internally produced and externally administered cytokines remains unclear. For example, it is not possible to exclude the possibility that increased levels of cytokines induced by bsAb have direct anti-tumor effects, although studies in the 38C13 model demonstrating the outgrowth of antigen-negative tumor suggests this is not the case (antigen-negative cells were not significantly affected by bsAb).[41] More likely, cytokines contribute to T-cell activation, which in turn enhances the cytotoxic potential of cells coated with bsAb. The role of bsAb Fc in this process also remains unclear. Further evaluation of different bsAb preparations will have important implications on selection of bsAb structure that is likely to be most effective clinically. If the bsAb-FcR interaction, and its resulting induction of T-cell activation, is required for effective bsAb-induced destruction of tumor, then intact bsIgG may be required. However, T-cell activation may be accomplished by the use of other T-cell activators that can be separately controlled, such as IL-2 or antibodies that induce activation via costimulatory molecules such as CD28. In such circumstances, bispecific $F(ab')_2$ antibodies, or genetically constructed bispecific peptides may be preferable to bispecific IgG.

A number of other questions remain. The ideal target antigen has not yet been identified. In particular, it is not clear whether bsAbs based on pan B-cell antibodies, such as anti-CD19, will be effective with acceptable toxicity, or whether molecules expressed more exclusively by malignant B-cells will need to be targeted. It also remains unclear whether bsAb induces circulating T-cells to target to tumor, or whether most of the anti-tumor effect results from T-cells that are already in the tumor are coated with bsAb in situ and induced to destroy the malignant cells. Such a distinction will help determine which histologies of B-cell malignancies are most likely to respond to bsAb therapy. If bsAb acts on T-cells that are already within the malignant tissue, then B-cell malignancies that are naturally infiltrated with T-cells are most likely to respond. If bsAb can actually induce circulating T-cells to target to tumor, then malignancies that contain mainly malignant cells may respond as well. Under such circumstances, the administration of adoptively transferred T-cells along with bsAb may be worth exploring. Ongoing studies in both mice and humans are evaluating malignant tissue for the presence of infiltrating lymphocytes. Evaluation of whether bsAb can induce T-cells to target to tumor is also being explored.

In conclusion, preliminary evidence in both animal models and clinical trials suggests bsAbs can retarget T-cells toward B-cell malignancies. Work in the laboratory and the clinic over the next few years should clarify the promise and limitations of this potentially powerful new form of immunotherapy.

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Animal cell culture

a practical approach

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CHAPTER 8

Cytotoxicity and Viability Assays

ANNE P. WILSON

1. INTRODUCTION

Drug development programmes for the identification of new cancer chemotherapeutic agents involve extensive pre-clinical evaluation of vast numbers of chemicals for detection of anti-neoplastic activity. The safety evaluation of compounds such as drugs, cosmetics, food additives, pesticides and industrial chemicals necessitates the screening of even greater numbers of chemicals. Animal models have always played an important role in both contexts, and although cell culture systems have figured largely in the field of cancer chemotherapy, where the potential value of such systems for cytotoxicity and viability testing is now widely accepted, there is increasing pressure for a more comprehensive adoption of *in vitro* testing in both spheres of application. The impetus for change originates firstly from financial considerations, since *in vitro* testing has considerable economic advantages over *in vivo* testing. Secondly, it occurs from a realisation of the limitations of animal models in relation to man as increasing numbers of metabolic differences between species come to be identified. Finally, it occurs from the moralistic viewpoint in terms of reducing animal experimentation.

The safety evaluation of chemicals involves an extensive range of studies on mutagenicity, carcinogenicity, teratogenicity and chronic toxicity, all of which are outside the scope of this text, but an obvious area of overlap between cancer chemotherapy and safety evaluation is that of acute toxicity. A precedent has been set with the volume of research carried out in the last 40 years in cancer chemotherapy, and those systems which have been found to be most relevant in this field must be of interest both in general toxicity and anti-neoplastic activity.

The fundamental requirements for both applications are similar. Firstly, the assay system should give a reproducible dose-reponse curve with low inherent variability over a concentration range which includes the *in vivo* exposure dose. Secondly, the selected response criterion should show a linear relationship with cell number and thirdly, the information obtained from the dose-response curve should relate predictively to the *in vivo* effect of the same drug.

2. BACKGROUND

Use of *in vitro* assay systems for the screening of potential anti-cancer agents has been prevalent since the inception of clinical cancer chemotherapy in 1946, following the discovery of the anti-neoplastic activity of nitrogen mustard. A number of reviews describe the historical development of these techniques and their applications (1,2) and more recent publications update to the present situation (3–5). The situation pertaining

in the field of safety evaluation of drugs has also been reviewed in the publication of a recent symposium convened by the Humane Research Trust (6).

Early cytotoxicity studies were largely qualitative, in that explant cultures growing in undefined medium were used for the study of drug effect, which could be 'quantitated' by assessment of either morphological damage or of inhibition of the zone of outgrowth. The development of a semi-defined growth medium, together with techniques for growing dispersed cells as a monolayer on glass, allowed the screening of identical replicate cell samples in reproducible growth conditions, which therefore meant that drug effects could be quantitated in a meaningful way. Using these techniques in conjunction with measurement of protein content of treated and untreated cells, Eagle and Foley were able to demonstrate a clear-cut correlation between *in vitro* and *in vivo* activity of neoplastic agents (7) demonstrating the validity of the method. The assay system was subsequently included in the Cancer Chemotherapy National Service Centre (CCNSC) screening programme and currently plays an essential role in pre-screening during the step-wise purification of natural fermentation beers, because its rapidity, minimal requirement for test compound and economy offer advantages which cannot be found in the *in vivo* screening systems (3).

Accumulated experience, both clinically and experimentally, demonstrated that heterogeneity of chemosensitivity existed between tumours, even those of identical histology. The successful development of the *in vitro* agar plate assay for antibiotic screening precipitated interest in the development of an analogous technique for 'tailoring' chemotherapy to suit the individual tumour and patient, thus removing the undesirable combination of ineffective chemotherapy in the presence of non-specific toxicity. The idea was first applied experimentally by Wright *et al.* (8) using explants of human tumor tissue and this report has been succeeded by numerous others, which aim to investigate the correlation between *in vitro* and *in vivo* results in humans. The methodology which has been used is varied and represents the multiplicity of factors which must be considered in devising these assays. In spite of this diversity the consensus of the majority of reports is that more than 90% positive correlation can be expected between *in vitro* resistance and clinical resistance, and approximately 60% positive correlation between *in vitro* sensitivity and clinical response. There is also an indication that the frequency of responding cultures *in vitro* is similar to the frequency of responding tumours *in vivo* (9,10).

The relationship between *in vitro* drug sensitivity exhibited by primary cultures of human tumours and their *in vivo* counterparts argues for their use in drug evaluation programmes since they provide a closer approximation to the human clinical situation than do the limited number of cell lines which are currently used. The role of the 'Human Tumour Stem Cell Assay' is under investigation in this context (11).

In vitro culture systems are also used extensively for mechanistic studies on drug action for which the same technical considerations apply, although more specific biochemical end-points may be required.

3. SPECIFIC TECHNIQUES

Decision making on the final choice of assay is a function of the context in which the assay is to be used, the origin of the target cells and the nature of the test compound.

Parameters which vary between different assays include:

(i) culture method;
(ii) duration of drug exposure;
(iii) duration of recovery period after drug exposure;
(iv) end-point used to quantitate drug effect.

3.1 Culture Methods

The choice of culture method depends on the origin of the target cells and the duration of the assay and, to some extent, dictates the end-point.

3.1.1 *Organ Culture*

The advantages offered by organ culture relate to the maintenance of tissue integrity and cell-cell relationships *in vitro*, thereby giving a closer analogy to the *in vivo* situation than the majority of other culture methods available. Reliable quantitation of drug effect is not facilitated by difficulties associated with size variation between replicates and cellular heterogeneity. Although the method has been used extensively to study the hormone sensitivity of potentially responsive target tissues, the number of studies relating to drug sensitivity is limited. The topic has been covered in a recent review article (12) (see also Chapter 7).

3.1.2 *Spheroids*

Spheroids result from the spontaneous aggregation of cells into small spherical masses, which grow by proliferation of the component cells. Their structure is analogous to that of a small tumour nodule, and the use of spheroids for drug sensitivity testing therefore permits an *in vitro* analysis of the effects of three-dimensional relationships on drug sensitivity, without the disadvantages previously mentioned for organ culture. Specific parameters which can be studied are:

(i) drug penetration barriers in avascular areas;
(ii) the effects of metabolic gradients (e.g., pO_2, pCO_2);
(iii) the effects of proliferation gradients.

The majority of studies have been carried out using spheroids derived from cell lines, but primary human tumours also have the capacity to form spheroids in approximately 50% of cases; the spheroid-forming capacity of normal cells is limited in comparison with tumour cells which is an important consideration since stromal elements may be excluded during reaggregation of human tumour biopsy material. Culture times in excess of 2 weeks are usually necessary for drug sensitivity testing, and the method is not suitable therefore in a situation where results are required quickly.

3.1.3 *Suspension Cultures*

(i) *Short-term cultures (4–24 h).* The short-term maintenance of cells in suspension for assay of drug sensitivity is applicable to all cell sources. When the cells are derived from human tumour biopsy material the assay system has several theoretical advantages in that the ability to grow is not a limiting factor since no growth is required, and cell overgrowth and clonal selection are minimised and results can be obtained

which is important if the assay is to be used in a clinical context. The method has been used extensively in West Germany for chemosensitivity studies on a variety of tumour types (13,14). A modified method using either tissue fragments or cells has been described by Silvestrini *et al.* (10) again with a variety of tumour types. Both groups used the incorporation of tritiated nucleotides into DNA/RNA as an end-point. Limitations of the method relate mainly to the short time period of the assay which precludes long drug exposures over one or more cell cycles and also takes no account of either the reversibility of the drug's effect or of delayed cytotoxicity. The type of drug which can be tested is therefore theoretically restricted, and although one group have found the main value of the test to lie with adriamycin (14), the successful use of other drugs with differing modes of action has been reported (10).

(ii) *Intermediate duration (4–7 days).* Suspension cultures of intermediate duration are particularly suited to chemosensitivity studies on haematological malignancies and have been described in several recent reports (15–17).

3.1.4 *Monolayer Culture*

The technique of growing cells as a monolayer has been most frequently applied to the cytotoxicity testing of cell lines, but the method has also been used with some success for studies on the chemosensitivities of biopsies from a variety of different tumour types. In the case of human biopsy material the greatest problems associated with the method are, firstly, that the success rate is limited because adherence and proliferation of tumour cells is not always obtained and, secondly, that contamination of tumour cell cultures by stromal cells (fibroblastic or mesothelial) is an all too frequent occurrence. The problem is greater with some tumour types than others (e.g., high for carcinoma of the breast, moderate for ovarian tumours and minimal for gliomas).

Some method of cell identification is therefore an essential part of such assays. Stromal cells appear to be more resistant to chemotherapeutic agents, but deliberate contamination of an ovarian tumour line (OAW 42) with up to 30% stromal cells had a minor effect on the chemosensitivity of the culture, implying that the majority of the measured response derived from the tumour cells. Heavier contamination with stromal cells gave intermediate values of sensitivity approximately as predicted. There was no evidence for the stromal cells conferring resistance on the tumour cells (*Figure 1*; Wilson, unpublished results). Similar results were obtained using drugs selected from antibiotics, anti-mitotics, alkylating agents and anti-metabolites.

The culture method probably offers the greatest flexibility in terms of possible drug exposure and recovery conditions, and also in methods of quantitation of drug effect. Of all methods described, the growth of cells in monolayers requires the lowest cell numbers, and it is therefore amenable to microscale methodology which permits multiple drug screening over a wide concentration range, and also facilitates automation. When cell numbers are really sparse it is often feasible to culture the cells until sufficient numbers are available for assay, although reports on changes in chemosensitivity after subculture are conflicting. Two to three subcultures are probably acceptable and, indeed, subculture has been recommended because variability between replicates is reduced (18). When stromal cell contamination is unacceptably high, subculturing also offers the possibility of 'purifying' tumour cell cultures by differential enzyme treatment or physical cell separation (see Chapters 5 and 6).

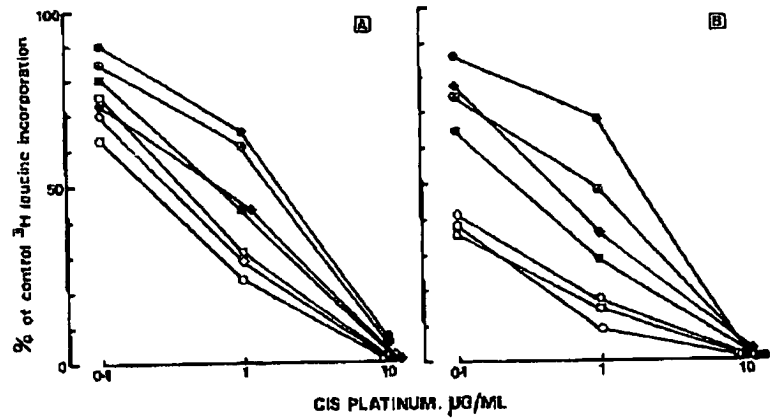

Figure 1. The effect of different proportions of mesothelial cells (A) and fibroblasts (B) on the measured sensitivity of an ovarian tumour cell line (OAW 42) to *cis*-platinum. (A.P.Wilson, unpublished results.) 10% stromal cells, □; 30% stromal cells, △;50% stromal cells, ■; 70% stromal cells, ●90% stromal cells, ●; 100% OAW 42, ○; 100% stromal cells, ⊗. Chemosensitivity was determined using the microtitration plate assay described in Section 9.4.2.

3.1.5 *Clonogenic Growth in Soft Agar*

Although monolayer cloning can be applied to cells cultured directly from the tumour, the majority of reports in recent years have used suspension cloning to minimise growth of anchorage-dependent stromal cells. Clonogenic assays have the theoretical advantage that the response is measured in cells with a high capacity for self-renewal (potentially the stem cells of the tumour) and cells with limited proliferative capacity, which make up much of the bulk of the tumour, are not assayed. However, this is only true if colonies are truly clones (i.e., were initiated from one cell and not from a clump) and are only scored after many cell generations in clonal growth. Regrettably this is often not the case. Cloning efficiencies of 0.01–0.1% are often quoted where it may be difficult to exclude the possibility of clumps, and while 10 generations (~1000 cells) may be readily obtained in monolayer cloning, suspension colonies are often scored after 4–6 cell generations (16–64 cells). Given that some of these colonies started out as clumps of 3–4 cells, the generation number may be as low as two and their capacity for self-renewal still be in some considerable doubt. Nevertheless, growth in soft agar is undoubtedly a useful assay system for cell lines which have a comparatively high plating efficiency. However, a number of technical problems have been encountered using solid tumours and effusions from patients which unfortunately influence interpretation of results. These include: difficulties in obtaining a pure single cell suspension from epithelial tumours (which is an essential requisite for the definition of clonogenic growth); very low plating efficiencies (<1%); the formation of colonies from anchorage-dependent cells under certain growth conditions; requirements for large cell numbers; and finally the somewhat subjective nature of colony quantitation. These represent a failure of present technology rather than a failure of the assay method, and the importance

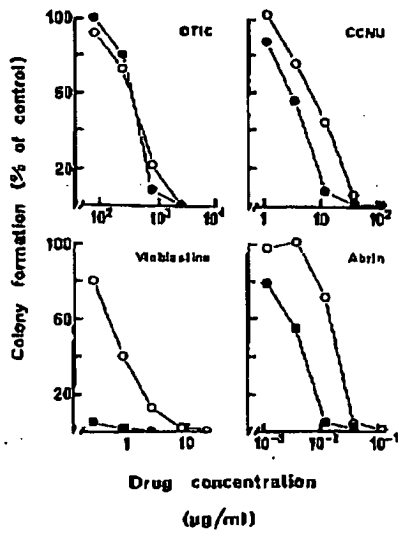

Figure 2. Dose-response curves of a melanoma xenograft (V.N.) cultured in soft agar using either (a) the 'Courtenay' method (○) or (b) the 'Hamburger-Salmon' method (●). Cells were exposed to the drugs for 1 h, plated at 3 x 10^4 cells per tube or dish and scored for colonies after 14 days incubation. Control cultures showed ~400 colonies using both methods. Method (b) showed greater sensitivity with three of the four drugs tested. (Reproduced with permission of the publishers. 26.)

of optimising methods of disaggregation and selective growth media for different tumour types has been emphasised (19). Critical assessment of the technical difficulties which have been encountered with the 'Human Tumour Stem Cell Assay' can be found in several reports (20–22). Results obtained using the double-agar method developed by Hamburger *et al.* (23) have been described in a recent review publication (24). An alternative methodology, developed by Courtenay and others (25), gives higher plating efficiencies, and has been compared with the 'Hamburger-Salmon' system by Tveit *et al.* (26), in which comparative study it was apparent that the methodology used influenced the chemosensitivity profile obtained (*Figure 2*).

4. DRUG CONCENTRATIONS

The choice of drug concentrations should be dictated by consideration of the therapeutic levels which can be achieved with clinically used drug dosages. When the compound is undergoing pre-clinical screening for potential activity this is not possible and, in the face of accumulated evidence on effective *in vitro* levels of compounds with known *in vivo* activity, an upper limit of 100 μg/ml is recommended. Pharmacokinetic data are available for many of the clinically used drugs, and parameters which are relevant to *in vitro* assays include the peak plasma concentration and the plasma clearance curves (*Figure 3*). A detailed description of pharmacological considerations may be found elsewhere (27). Pharmacokinetic data on most cancer chemotherapeutic agents, which

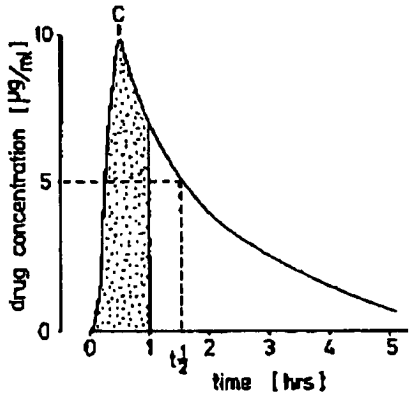

Figure 3. Typical plasma-clearance curve for intravenously administered drug. C = peak plasma concentration. $t_{\frac{1}{2}}$ = terminal half-life of drug in plasma. ⊠ = area under curve for T = 1 h (μg/ml/h).

Table I. A Comparison of Theoretical[a] Plasma Levels with Pharmacokinetic Data (28) for a Range of Cancer Chemotherapeutic Agents.

Drug	*Dosage*	*Theoretical plasma level μg/ml*	*Measured C × T μg/ml/h*
Melphalan	0.6 mg/kg	0.86	0.87–5.50
Cytosine arabinoside	10 mg/kg	14.29	15.23
BCNU	2.25 mg/kg	3.21	1.02
5-Fluorouracil	15 mg/kg	21.43	13–21
cis-platinum	100 mg/m² (e.g., S.A. = 1.6)	3.8	1.42–2.52
Vinblastine	0.2 mg/kg	0.29	0.116–0.254

[a]The calculation is based on a 60 kg person with a 70% fluid compartment.

includes peak plasma concentration, the C × T parameter (where C = concentration and T = time in hours, and the terminal half-life ($t_{\frac{1}{2}}$) of the drug in plasma has been summarised (28). When no pharmacokinetic data are available, an approximation of the plasma levels can be obtained by calculation of the theoretical concentration obtained when the administered dose is evenly distributed throughout the total body fluid compartment (*Table I*). It is axiomatic that the concentration range adopted should give a dose-response curve, and the range selected by different groups reflects the different sensitivity levels of the various assays.

5. DURATION OF DRUG EXPOSURE

Pharmacokinetic data show that maximum exposure to drug occurs in the first hour after i.v. injection and, for this reason, an exposure period of 1 h has been chosen by many investigators. Whilst this may be adequate for cycle-specific drugs, such as the alkylating agents, longer exposure times over several cell cycles are necessary for phase-

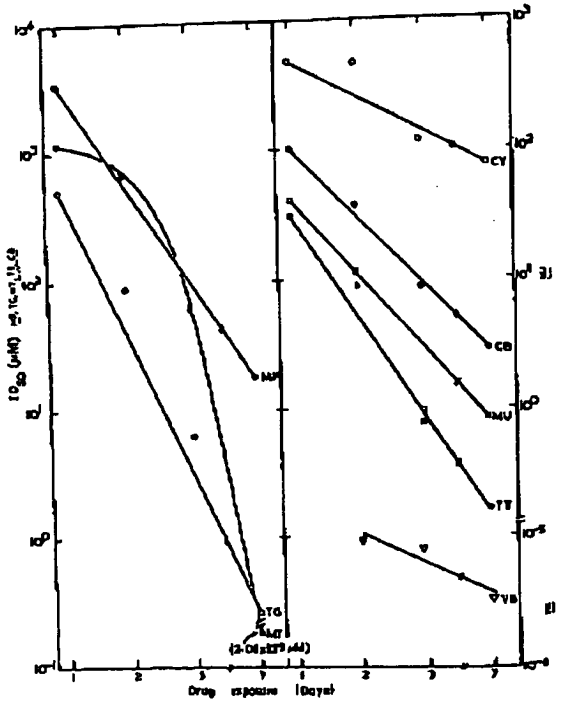

Figure 4. Effect of prolonged drug exposure on ID_{50} values for 6-mercaptopurine (MP), thioguanine (TG), methotrexate (MT), cyclophosphamide (CY), chlorambucil (CB), mustine (MU), thiotepa (TT) and vinblastine (VB), tested against HeLa cells using a microtitration plate assay (Section 9.4.2). Drugs were replaced at 24-h intervals for the 48-h and 72-h exposures, and at 24, 48 and 72 h for the 7-day exposure. A break in the time-axis is therefore shown between 3 and 7 days. (Reproduced with permission of the publishers, 9.)

pecific drugs such as methotrexate and vinblastine. However, prolonged drug exposure sing a variety of cancer chemotherapeutic agents has been shown to result in gradually ecreasing ID_{50} values, as exposure times increase (29) (*Figure 4*). Rate of penetration f the drug may also be a limiting factor when short exposure times are used. The level f cell kill achieved with a short exposure time is also related to the method of assay; hilst high levels of cell kill can be obtained with a 1 h exposure to an alkylating agent sing primary suspension cloning, a similar duration is insufficient to show cytotoxicity monolayer.

Ultimately the question of duration of drug exposure becomes one of practicality. a significant effect is achievable in 1 h, then this should be used. Many drugs may ind irreversibly to intracellular constituents and the actual exposure may therefore e in excess of 1 h due to drug retention. Others, principally the anti-metabolites and nti-tubulins, are more likely to be reversible if not present at the sensitive phase of

Table 2. Stability of Some Cancer Chemotherapy Drug Solutions at 37°C.

Drug	*Stability data*	*Reference*
Adriamycin	Stable during 48 h pre-incubation	A.P. Wilson (unpublished results)
Bleomycin	Stable during 48 h pre-incubation	
5-Fluorouracil	Stable during 48 h pre-incubation	
Cytosine arabinoside	Stable during 48 h pre-incubation	
cis-platinum	Full activity retained for 6 h, complete loss at 48 h	(29)
Phosphoramide mustard	Full activity retained for 24 h, some loss at 48 h	
Melphalan	Full activity retained for 1 h, complete loss at 24 h.	
Chlorambucil	6-fold decrease in ID_{50} after 24 h pre-incubation	
Mustine	25-fold decrease in ID_{50} after 24 h pre-incubation	
Thiotepa	1.38-fold decrease in ID_{50} after 24 h pre-incubation	
Cyclophosphamide	3.5-fold decrease in ID_{50} after 24 h pre-incubation	
Vinblastine	No change in ID_{50} after 24 h pre-incubation	

the cell cycle, and prolonged exposure spanning one or more cell cycles may be required. Resistance of the surviving fraction when short exposures are used may be due to an inappropriate phase of the cell cycle during drug exposure but truly resistant cells (i.e., with resistance even at the appropriate phase of the cell cycle) can only be demonstrated unequivocally after a prolonged exposure.

Regardless of the exposure time selected, it must be kept constant for each drug where tumours are being compared, and constant between drugs where two similar compounds are being compared. When prolonged drug exposure times are used, it should be remembered that the theoretical C × T value is only equal to the actual C × T value when the drug regains full activity at 37°C over the entire exposure period and the response is linear with time. Data on the stability of some drug solutions at 37°C are summarised in *Table 2*. The effective concentration of drug may also be reduced by binding of drug to the surface of the incubation vessel; this has been noted with adriamycin and actinomycin D.

6. RECOVERY PERIOD

The inclusion of a recovery period following drug exposure is important for three reasons:

(i) when metabolic inhibition is used as an index of drug-effect; it allows recovery of metabolic perturbations which are unrelated to cell death;

(ii) sub-lethal damage can be repaired;

(iii) delayed cytotoxicity, such as occurs with 6-mercaptopurine and methotrexate, can be expressed.

Depending upon the nature of the drug and the end-point of the assay, absence of a recovery period can either under-estimate or over-estimate the level of cell kill achieved. However, it is equally important that the recovery period is not too long, because cell kill can be masked by overgrowth of a resistant population. In monolayer assays which monitor cell counts or precursor incorporation, the cells must remain in the log phase of growth throughout the exposure and recovery period. In clonogenic assays the recovery period is the period of clonal growth; the time taken to form measurable colonies is a minimum of five or six cell generations (32—64 cells/colony) in suspension assays and usually much greater than this when monolayer cloning is in use.

2. END-POINTS

2.1 Cytotoxicity, Viability and Survival

Interpretation of the significance of assay results is dependent upon distinguishing between assays which measure cytotoxicity and assays which measure cell survival. Cytotoxicity assays measure drug-induced alterations in metabolic pathways or structural integrity which may or may not be related directly to cell death, whereas survival assays measure the end-result of such metabolic perturbations which may be either cell recovery or cell death. Theoretically, the only reliable index of survival in proliferating cells is the demonstration of reproductive integrity, as evidenced by clonogenicity. Metabolic parameters also may be used as a measure of survival when the cell population has been allowed time for metabolic recovery following drug exposure.

When the test compound exhibits non-specific toxicity (i.e., toxicity which is not specifically related to proliferative potential) resulting in loss of one or more specific and essential cell functions rather than loss of reproductive capacity, a cytotoxicity test may be more appropriate.

2.2 Cytotoxicity and Viability

Some cytotoxicity assays offer instantaneous interpretation, such as the uptake of a dye by dead cells, or the release of ^{51}Cr or fluorescein from pre-labelled cells. These have been termed tests of viability and are intended to predict survival rather than measure directly. On the whole these tests are good at identifying dead cells but may overestimate long-term survival. Most imply a breakdown in membrane integrity and irreversible cell death.

Other aspects of cytotoxicity, measuring metabolic events, may be more accurately quantified and are very sensitive, but prediction of survival is less certain as many forms of metabolic inhibition may be reversible. In these cases impairment of survival can only be inferred if depressed rates of precursor incorporation into DNA, RNA or protein are maintained after the equivalent of several cell population doubling times has elapsed.

2.1 *Membrane Integrity*

This is the commonest measurement of cell viability at the time of assay. It will give an estimate of instantaneous damage (e.g., by cell freezing and thawing), or progressive damage over a few hours. Beyond this, quantitation may be difficult due to loss of dead cells by detachment and autolysis.

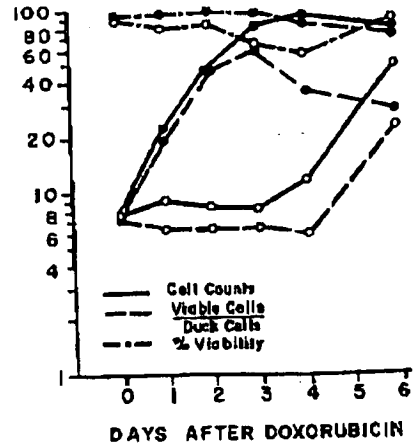

Figure 5. Effect of doxorubicin (0.12 μg/ml/h) on MDAY-D2 cells as assessed by three different techniques: Coulter counter particle counts (——) (Y axis units are cells/ml x 10^{-5}); ratio of living tumour cells to duck red blood cells, normalised to the same scale as the Coulter counts (- - - -), and % viability (— · —) Y axis units are % viability (living cells/living and dead cells x 100%). ● = control cultures; ○ = doxorubicin-treated cultures. (Reproduced with permission of the publishers, 35.)

(i) *^{51}Cr release.* Labelling cells with ^{51}Cr results in covalent binding of chromate to basic amino acids of intracellular proteins. These labelled proteins leak out of the cell when the membrane is damaged, at a rate which is proportional to the amount of damage incurred. The method is used extensively in immunological studies for determining cytotoxic T cell activity against tumour target cells. Natural leakage of ^{51}Cr from undamaged cells is high and therefore the time period over which the assay can be used is restricted to approximately 4 h. In one comparative study which evaluated ^{51}Cr release as an end-point for drug cytotoxicity testing the method was found to be of no value (34).

(ii) *Dye exclusion.* Viability dyes which have been used to determine membrane integrity include trypan blue, eosin Y, naphthalene black, nigrosin (green), erythrosin B and fast green. Staining for viability assessment is more suited to suspension cultures than monolayers, because dead cells detach from the monolayer and are therefore lost from the assay. A major disadvantage is the failure of reproductively dead cells to take up dye, as was demonstrated when cells with impaired clonogenicity showed 100% viability according to dye exclusion (34). The method has been renovated however, and technical innovations introduced which attempt to circumvent some of the problems commonly associated with such assays. In the methodology developed by Weisenthal *et al.* (35), a 4-day assay period is used to permit reproductively dead cells to lose their membrane integrity and the inaccuracies produced by either overgrowth of viable cells or lysis of dead cells is compensated for by incorporation of fixed duck erythrocytes as an internal standard. Comparison of cell counts *versus* % viability *versus* viable cell/duck cell ratio demonstrated the increased sensitivity of the latter method (*Figure 5*). The method has

been applied with equal success to solid tumours, effusions and haematological malignancies.

7.2.2 *Respiration and Glycolysis*

Drug-induced changes in respiration (O_2 utilisation) and glycolysis (CO_2 production) have been measured using Warburg manometry, both parameters showing dose-related depression (31,32). Other authors have determined dehydrogenase activity by incorporating methylene blue into agar containing drug-treated cells, cell death being indicated by non-reduction of the dye (33). The latter method has the disadvantage of being non-quantitative, whilst the former, although quantitative, has not been widely adopted because the technical manipulations involved are extensive and unsuited to multiple screening.

7.2.3 *Radioisotope Incorporation*

Measurement of the incorporation of radiolabeled metabolites is a frequently used end-point for cytotoxicity assays of intermediate and short-term duration.

(i) *Nucleotides.* Measurement of [^{3}H]thymidine incorporation into DNA and [^{3}H]uridine incorporation into RNA are two of the most commonly used methods of quantitation of drug cytotoxicity (10, 13, 14, 16). In short-term assays, which do not include a recovery period, there are a number of disadvantages, all of which relate to a failure of [^{3}H]thymidine incorporation to reflect the true DNA synthetic capacity of the cell. These are:

i) changes may relate to changes in size of the intracellular nucleotide pools rather than changes in DNA synthesis;

ii) some drugs such as 5-fluorouracil and methotrexate which inhibit pyrimidine biosynthesis (*de novo* pathway) cause increased uptake of [^{3}H]thymidine due to a transfer to the 'salvage' pathway, which utilises pre-formed pyrimidines;

iii) continuation of DNA synthesis in the absence of [^{3}H]thymidine incorporation can occur (36).

The choice of isotope appears, to some extent, to be dependent on the drug; for example, Volm *et al.* (13) have recommended [^{3}H]uridine for adriamycin, [^{3}H]thymidine for 4-hydroxperoxycyclophosphamide and [^{3}H]deoxyuridine for 5-fluorouracil, whilst Silvestrini *et al.* (10) reported similar uptake of [^{3}H]thymidine and uridine for adriamycin, 4-hydroperoxycyclophosphamide, cis-platinum and mitomycin C, but differing uptake for bleomycin, vincristine, actinomycin D and VP-16. The low labelling index of human tumours with resultant low levels of nucleotide incorporation in short-term assays necessitates the use of high cell densities which can restrict the number of drugs and range of concentrations tested when cell numbers are limited. Two different 'hybrid' techniques have recently been reported, which seek to combine the 'stromal cell inhibition' advantage offered by the soft agar culture system with the facilitated quantitation offered by the use of radioisotopes. Both assays are of intermediate duration (~4 days) and use [^{3}H]thymidine incorporation into DNA as an end-point; in one method the cells are grown in liquid suspension over soft agar (37), whilst in the other the cells are incorporated in the soft agar (38).

Given that a homogeneous cell population is available, [^{3}H]nucleotide incorporation

can be used after an appropriate recovery period to measure survival or, in the presence of drug, to measure an anti-metabolic effect, but with the reservations expressed above.

(ii) [^{125}*I*]*Iododeoxyuridine* ([^{125}*I*]*UdR*). [^{125}I]UdR is a specific, stable label for newly synthesised DNA which is minimally re-utilised and can therefore be used over a 24-h period to measure the rate of DNA synthesis (39); quantitation is facilitated because the isotope is a gamma emitter. Disadvantages include its variable toxicity to different cell populations, which therefore means that more cells are required because [^{125}I]UdR must be used in low concentrations.

(iii) [^{32}*P*]*Phosphate* (^{32}*P*). The rate of release of ^{32}P into the medium from pre-labelled cells is a function of the cell type and is increased in damaged cells. This has been used as a measure of drug efficacy (40). The incorporation of ^{32}P into nucleotides has also been used as an index of drug cytotoxicity (41). Neither method has been routinely adopted.

(iv) [^{14}*C*]*Glucose.* Glucose incorporation is used as a cytotoxicity end-point because it is a precursor which is common to a number of biochemical pathways (42). The method has not been widely used.

(v) [^{3}*H*]*Amino acids.* Protein synthesis may be considered as an essential metabolic process without which the cell will not survive, and incorporation of amino acids into proteins has been used successfully as an index of cytotoxicity. The most extensive studies have utilised monolayers of cells growing in microtitration plates, using either incorporation of [^{3}H]leucine (29) measured by liquid scintillation counting, or [^{35}S]-methionine incorporation, measured using autofluorography (43).

7.3 Survival (Reproductive Integrity)

Survival assays give a direct measure of reproductive cell death by measuring cloning efficiency either in monolayer or in soft agar. The end-points which have been described in the previous section can also be used as an index of reproductive integrity when the design of the assay incorporates a recovery period. Increases in total protein, cell number or protein synthetic capacity have been taken to imply proliferative ability although interpretation is more difficult due to differential responses in elements of a heterogeneous cell population.

7.3.1 *Cloning in Monolayer*

Cells generally have a higher cloning efficiency in monolayer than they do in soft agar, and the method is frequently used for cell lines. Normal cells and tumour cells will form colonies in monolayer, and the method is not therefore applicable to tumour biopsy material, which commonly shows high levels of stromal cell contamination, unless criteria are available to discriminate between tumour and stroma. Feeder layers of irradiated or mitomycin C-treated cells can be used to increase plating efficiencies, and indeed small drug-resistant fractions are more likely to be detected in the improved culture conditions existing when feeder cells are used (44).

7.3.2 *Cloning in Soft Agar*

The advantages and disadvantages of this method have been discussed in Section 3.1.5.

7.3.3 *Spheroids*

Various methods can be used to quantitate the effect of drugs on spheroidal growth. These are:

(i) relative changes in volume of treated and untreated spheroids;
(ii) cloning efficiency in soft agar of disaggregated spheroids;
(iii) cell proliferation from spheroids adherent to culture surfaces.

The first method is rather insensitive because spheroidal growth tends to plateau, and the second may be affected by difficulties with disaggregation to a single cell suspension and low plating efficiencies. The choice of end-point is largely a function of the individual characteristics of the particular spheroids under study.

7.3.4 *Cell Proliferation*

An increase in cell number in a proliferating cell line can be regarded as an index of normal behaviour. Growth curves may be determined and the doubling time during exponential growth derived. An increase in the doubling time is taken as an indication of cytotoxicity, but it must be stressed that this is a kinetic measurement averaged over the whole population and cannot distinguish between a reduced growth rate of all cells and an increase in cell loss at each cell generation. It must be emphasised that estimates of cytotoxicity based on cell growth in mass culture must utilise the whole growth curve, or they may be open to misinterpretation. If 50% of cells die at the start of the experiment, the growth rate of the residue, determined in log phase, may be the same, but will show a delay. In practice it is very difficult to distinguish between early cell loss and a prolonged log period where cells are simply adapting. For these reasons, cell growth rates must be taken only as a rough guide to cytotoxicity, and accurate measurements of cell survival and cell proliferation should be made by colony-forming efficiency (survival) and colony size (proliferation).

7.3.5 *Total Protein Content*

Protein content determination is a relatively simple method for estimating cell number. It is particularly suited to monolayer cultures, and has the advantage that washed samples can be stored refrigerated for some time before analysis without impairment of results, facilitating large-scale screening. Over-estimation of cell number may arise with some drugs which inhibit replication without inhibiting protein synthesis (e.g., 5-bromodeoxyuridine, methotrexate). An adaptation of the Lowry method has been specifically developed for monolayer cultures (30), using the Folin-Ciocalteau phenol reagent. An alternative method using amido black has also been recommended because the linear section of the standard curve extends over a wider concentration range (3). Assessment of cytotoxicity by this method requires the demonstration of an alteration in the accumulation of protein per culture against time, preferably taken at several points, or at one point after prolonged drug exposure and recovery, as described above.

8. ASSAY COMPARISONS

In spite of the diversity of methodologies used for cytotoxicity and viability testing, the same levels of correlation between *in vitro* sensitivity and *in vivo* response have been reported when the various methods have been used on human tumour biopsy material. A number of comparative studies have been undertaken, in which clonogenicity has been taken as the standard for comparison. A summary of the results is shown in *Table 3*. It would appear that appropriately designed cytotoxicity assays give results comparable to clonogenic assays.

Table 3. A Summary of Studies Undertaken to Compare the Results of Clonogenic Assays with the Results of Cytotoxicity Assays.

Authors	*Methods compared*	*Cells used*	*Findings*
Roper and Drewinko (34)	^{51}Cr release; dye exclusion; labelling index; growth kinetics	T_1 lymphoma	Clonogenicity only reliable dose-dependent index of drug effect
Tveit *et al.* (26)	'Courtenay' *versus* 'Salmon' soft agar systems	Melanomas: xenografts and biopsies	Chemosensitivity results not comparable: 'Salmon' system shows greater sensitivity
Morgan *et al.* (18)	Monolayer cloning *versus* microtitration assay (Sections 9.4.2 and 9.4.3)	Human astrocytoma	Good correlation: cloning more sensitive in detection of small resistant fractions
Wilson *et al.* (45)	Short-term biochemical assay (Section 9.4.4) *versus* microtitration assay	Cell lines (T13 and MCF 7)	Comparability dependent on drug and exposure time. When not directly comparable, cut-off points selected from training data gave comparable results
Weisenthal *et al.* (17)	Dye exclusion	Animal tumour cell lines	Qualitative agreement between dye exclusion and clonogenic assay
Friedman and Glaubiger (37)	[^{3}H]TdR incorporation in liquid suspension over soft agar	Human tumour biopsy material	~89% correlation between [^{3}H]TdR incorporation and 'Human Tumour Stem Cell Assay

9. TECHNICAL PROTOCOLS

9.1 Drugs and Drug Solutions

9.1.1 *Drug Sources*

Pharmaceutical preparations for i.v. administration frequently contain various additives which may themselves be cytotoxic. Such preparations are therefore not suitable for aliquotting by weight and, if they are used as a stock solution, the cytotoxicity of the additional components should be determined in the assay system used. The problem can be avoided by obtaining pure compounds from the drug manufacturers.

9.1.2 *Storage*

It is recommended that dry compounds be stored at −20°C to −70°C over desiccant; this is especially important with compounds which are unstable in aqueous solution. It is routine practice to make up stock drug solutions which are then aliquotted and

Table 4. Solvents Used for Cancer Chemotherapy Drugs.

Soluble in aqueous solution	*Insoluble*	*Solvents used*
Adriamycin	Chlorambucil	2% HCl:98% ethanol diluted with 4.5 vols of propane 1,2-diol and 4.5 vols saline or DMSO[a]
Actinomycin D		
Bleomycin		
Mitomycin C		
Cytosine arabinoside		
5-Fluorouracil		
Methotrexate	Melphalan	0.1 M HCl or DMSO
Vinblastine		
Vincristine		
Thiotepa	BCNU	Ethanol
cis-platinum		
4-Hydroperoxycyclophosphamide	CCNU	Ethanol plus 1 vol each of 5% Tween 80 and PBS[b]
Procarbazine	Hexamethylmelamine	DMSO or homogenise in DMSO plus 9 vols 5% Tween 80 in saline
	6-Mercaptopurine	DMSO

[a]Dimethylsulphoxide.
[b]Phosphate buffered saline.

stored at −70°C. Storage at a higher temperature is not recommended, and some drugs (nitrosoureas) are unstable even under these conditions (46). As some drugs may bind to conventional cellulose nitrate or acetate filters, sterile filtration must be carried out under controlled conditions to check for binding of drug. Use the maximum drug concentration and maximum volume to saturate the filter within the first few ml of filtrate, which may then be discarded. Alternatively use non-absorbent filters, e.g. nylon. In either case the filtrate should be assayed to make sure no activity has been lost. In practice it has been found that handling of non-pharmaceutical preparations of pure drugs under aseptic conditions (i.e., use of sterile blade for weighing out into sterile container) is sufficient to prevent contamination, even in experiments of prolonged duration.

9.1.3 *Diluents*

(i) *Solvents.* Solvents which can be used for the common cancer chemotherapeutic agents are shown in *Table 4*. Ethanol should be diluted out 1000-fold to avoid cytotoxicity; dimethyl sulphoxide is not normally cytotoxic at 1/100 dilution, but some primary cultures of human tumours may show exquisite sensitivity to the compound. Solvent controls should therefore be routinely included when primary culture material is being assayed.

(ii) *Medium components.* Certain drugs bind avidly to serum proteins (e.g., *cis*-platinum), and the presence of serum in the incubation medium may therefore reduce the amount of available drug. When the sensitivity of cells to *cis*-platinum was determined using a 0, 5, 10 or 20% serum concentration, the influence of serum was found to depend on the exposure time (*Figures 6A* and *B*). Thus, for a 3-h exposure, cells were more

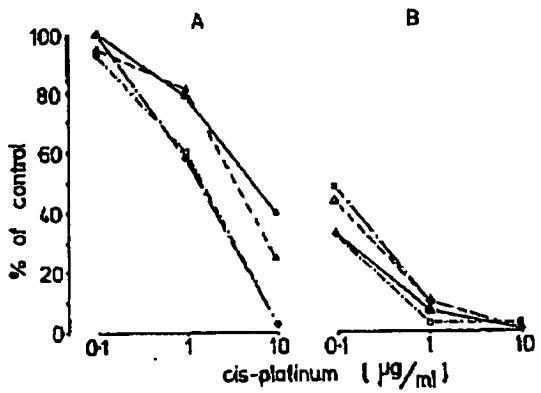

Figure 6. Effect of serum concentration on the sensitivity of an ovarian tumour cell line (OAW 42) to *cis*-platinum, measured using a microtitration plate assay (Section 9.4.2). Exponentially growing cells were exposed to *cis*-platinum for 3 h with a 72-h recovery period (A) or for 48 h with a 24-h recovery period (B). 0% serum, ○; 5% serum, ●; 10% serum, △; 20% serum, ▲. Standard deviations were ≤ ±10%. (A.P.Wilson, unpublished results.)

sensitive in the presence of low serum concentrations whilst consistent sensitivity in all serum concentrations was observed with a 48-h exposure period (A.P.Wilson, unpublished results).

Components of some media can protect cells against the cytotoxic effects of anti-metabolites (e.g., thymidine and hypoxanthine protect against methotrexate, thymidine protects against 5-bromodeoxyuridine and 5-FdUR). A comprehensive list has been detailed elsewhere (3). Such considerations are of particular importance when screening new agents for potential cytotoxic activity.

9.1.4 *Drug Activation*

Many compounds which are not themselves cytotoxic are converted to cytotoxic metabolites by the P450 mixed oxidase system of the liver. Cyclophosphamide is the best known of the cancer chemotherapeutic compounds to require *in vivo* activation, and methods of obtaining active metabolites have included use of urine from drug-treated patients, and also S9 microsome fractions prepared from the livers of phenobarbitone treated rats for *in vitro* activation. Pure preparations of phosphoramide mustard and 4-hydroperoxycyclophosphamide can now be obtained, and although the argument as to which of these compounds is responsible for *in vivo* anti-neoplastic activity is not fully resolved, experimental evidence seems to favour the 4-hydroperoxycyclophosphamide derivative (47).

Alternative methods for *in vitro* activation are under investigation using cultured rat hepatocytes or human liver biopsy material. The use of intact cells, in which the levels of co-factors resemble those *in vivo* and are high enough to sustain the associated reactions, provides a closer approximation to the *in vivo* situation, and indeed may give different results to those obtained using liver homogenates. Species differences exist

in the complement of cytochrome-P450 system found in the liver, and for this reason the use of human liver homogenate may again provide a closer analogy to the *in vivo* situation, especially since individual variations in metabolic activity also exist. The subject has been discussed in detail elsewhere (48,49).

9.2 Drug Incubation

It is common procedure to incubate cells with drug solutions immediately after enzyme disaggregation of solid tissue, or harvesting of cell monolayers by trypsinisation. There is evidence to suggest that susceptibility of cells to drug is altered by enzyme treatment and does not return to 'untrypsinised' levels until approximately 12 h after enzyme exposure (50). It may therefore be expedient to include a pre-incubation recovery period for freshly disaggregated cells to allow for this.

Maintenance of pH at 7.4 is essential during the incubation period since alterations in pH will alter cell growth and alkaline pH particularly will markedly reduce cell viability.

Comparison of static *versus* non-static incubations of cell suspensions revealed marked differences in dose-response profiles (51), and cells should therefore be kept in continuous suspension to allow equal drug distribution. If the surface area:depth ratio of the incubation vessel is small, incubation in a water-shaker bath will not keep the cells in suspension, and intermittent shaking by hand (e.g., at 10-min intervals) is recommended.

9.3 Assay by Survival and Proliferative Capacity

9.3.1 *Clonogenicity*

One of the most generally accepted methods for assaying for survival is the measurement of the ability of single cells to form colonies in isolation. This is usually achieved by simple dilution of a single cell suspension, and survival determined by counting the colonies which form. A lower threshold must be set in line with the doubling time of the cells being studied and the total duration of the assay. Five or six doublings (32 or 64 cells/colony) is usually taken as the lower threshold.

As some drugs may have an effect on cell proliferation as well as, or instead of, survival *per se*, it may be necessary to do a colony size analysis as well. This may be done by counting the number of cells per colony (very tedious and only possible in small colonies), by measuring the diameter (prone to error if cell size or degree of piling up changes) or by measuring absorbance of colonies stained with 1% crystal violet.

(i) *Monolayer cloning.* Adherent cells are plated onto a flat surface of glass or tissue culture-treated plastic, allowed to grow and form colonies, stained and counted. Drug treatment is best performed before subculture for plating, where highly toxic substances are being tested. For low grade toxins, where chronic application is required, they may be applied 24–48 h after plating, and retained throughout the clonal growth period, provided they are stable.

(1) Prepare replicate 25 cm^2 flasks, two for each intended concentration of drug, and two for controls.

(2) When cultures are at the required stage of growth (usually mid-log phase but, in special circumstances, may be in the plateau phase of the growth cycle) add the drug to the test and solvent to the control for 1 h at 37°C.

(3) Remove the drug, rinse the monolayer with phosphate-buffered saline (PBS), and prepare a single cell suspension by conventional trypsinisation (see Chapter 1).

(4) Count the cells in each suspension and dilute to the appropriate cell concentration to give 100–200 colonies per 5 or 6 cm Petri dish. This figure depends on the plating efficiency of the cells and the effect of the drug (e.g., control plates with no drug, from a cell culture of a known plating efficiency of 20%, will require 500–1000 cells per dish, at the ID_{90} of the drug, will require 1000–2000 cells per dish and at the ID_{10}, 5000–10 000 cells per dish). A trial plating should be done first:

(a) to determine the plating efficiency of the cells,

(b) to determine approximately the ID_{90} and ID_{10} of the drug.

In practice it is more usual to set up dishes at two cell concentrations, one to give a satisfactory number of colonies at low drug concentrations and controls, and one for higher drug concentrations, with some overlap in the middle range. Experience will usually determine where this is likely to fall.

(5) Plate out the appropriate number of cells per dish and place in a humid incubator at 37°C with 5% CO_2.

(6) Grow until colonies form. For rapidly growing cells (15–24 h doubling time) this will take 7–10 days, for slower growing cells (36–48 h doubling time) 2–3 weeks are required. In general, for a survival assay the colonies should grow to 1000 cells or more on average (10 generations). As the colonies increase in size the growth rate (particularly of normal cells) will slow down as the colonies tend to grow from the edge, and the slower growing colonies will tend to catch up. Hence if cell proliferation (colony size) is the main parameter, clonal growth should be determined at shorter incubation times giving smaller colonies with a wider size distribution.

(7) Rinse dishes with PBS, fix in methanol or glutaraldehyde and stain with 1% crystal violet, rinse in running tap water, distilled water and dry.

(8) Count colonies above threshold and calculate as a fraction of control. Plot on a log scale against drug concentration.

(ii) *Clonogenicity in Soft Agar Using a Double Layer Agar System.* The following procedure utilises a 1-h drug exposure period, and gives four replicates per test condition.

(1) Prepare 35 mm Petri dishes with a 1 ml base layer of 0.5% agar in growth medium by mixing 1% agar (melted by autoclaving or boiling) at 45°C with an equal volume of double strength medium at 45°C and dispensing 1 ml aliquots in a pre-heated pipette.

(2) Prepare a single cell suspension of the target cell population (see Chapters 1 and 6), adjust the cell concentration in growth medium to give 20 times the final concentration desired at plating and store at 4°C until ready to use.

(3) Prepare drug dilutions in growth medium and aliquot out 900 µl of each concentration into duplicate tissue-culture grade tubes, including control tubes contain-

ing growth medium only and appropriate solvent controls. Because of the time-span involved when plating out multiple drugs and concentrations, additional controls are recommended for plating out at the beginning, middle and end of setting up the experiment. The controls thus incorporate variability due to the time involved in setting up the assay.

(4) Check that the stock cell suspension still comprises single cells, and add 100 µl to each of the prepared tubes.

(5) Incubate the tubes at 37°C for 1 h (see Section 8.2).

(6) At the end of the incubation period centrifuge the tubes (2 min, 100 g) and wash the cells in 5 ml of saline. Repeat once more, and resuspend the cell pellet in 2 ml of growth medium. Keep the cells on ice to maintain cell viability whilst plating out replicates.

(7) Centrifuge the duplicate set of tubes for one drug concentration; remove medium and add 2 ml of warmed growth medium containing 0.3% agar to each tube. Needle gently to disperse cell aggregates and plate out 1 ml aliquots onto each of four prepared bases. The final plating out is most easily accomplished using a 1 ml micropipette (Finn pipette or equivalent): if ~2 mm is cut off the end of the tips this prevents problems due to blockage of the small aperture by solidified agar.

(8) Put the dishes to solidify on a cooled, horizontal surface.

(9) Repeat for all test conditions, including controls at appropriate intervals throughout.

(10) Incubate the plates in a humidified atmosphere of 95% air/5% CO_2.

(11) Score the plates for colonies when control colonies have reached a pre-determined size. This is usually more than 50 cells for cell lines, but a size of more than 30 or 20 cells has been used when the population has a slow growth rate and a low plating efficiency, as with human tumour biopsies.

(iii) *Modifications*

(1) *The 'Courtenay' method.* The 'Courtenay' method for suspension cloning (51) utilises rat red blood cells as feeder cells, and a 5% O_2 tension. The procedure outlined above may be used, with appropriate modification of final plating conditions.

(2) *Feeder cells.* A linear relationship between plating efficiency and plated cell numbers may not occur from low to high cell numbers. When cell lysis has occurred due to drug treatment, the reduction in cell numbers can reduce the plating efficiency disproportionately in relation to seeding density rather than reflecting the clonogenicity of the remaining cell population. This problem can be circumvented by incorporating homologous feeder cells in the assay which have either been lethally irradiated with a ^{60}Co or ^{137}Cs source, or treated for approximately 12 h with 2 µg/10^6 cells of mitomycin C. The radiation dose needs to be established for each cell type, (e.g., 2000–3000 rads for lymphocytes, 6000 rads for lymphoid cell lines.

3) *Use of (2-(p-iodophenyl)-3-(p-nitrophenyl)-5 phenyl tetrazolium chloride) (INT).* Viable cells reduce colourless tetrazolium salts to a water-insoluble coloured formazan product, and the reaction has been used to distinguish viable colonies

from degenerate clumps when scoring finally. It should be noted, however, that viable colonies may become degenerate due to nutrient deficiency which may cause misleading results. The stain is made by dissolving INT violet in buffered saline to a final concentration of 0.5 mg/ml; dissolution is slow and the stain needs to be prepared 24 h prior to use. Add 0.5–1 ml to each 35 mm Petri dish and incubate overnight at 37°C. Viable colonies then stain a reddish-brown colour.

(4) *20% O_2 versus 5% O_2.* The lower oxygen tension, which more closely resembles physiological levels of oxygen, is recommended for clonogenic assays because it results in higher cloning efficiencies. There is evidence to suggest that it also modifies the chemosensitivity profile of the cell population (52), producing enhanced cytotoxicity.

9.3.3 *Spheroids*

The experimental protocols outlined below are based on techniques described in a recent publication (53). Three end-points may be used to determine the cytotoxic effect of drugs on spheroids; these are: (a) volume growth delay; (b) clonogenic growth; and (c) outgrowth as a cell monolayer. Pre-selection of similarly sized spheroids and drug incubation is a common starting point.

(i) Pre-select spheroids in the chosen size range. Sizes in the range 150–250 µm are just visible to the naked eye and can be selected using a Pasteur pipette.

(ii) The method of incubation with drug depends to some extent on the exposure period to be used. For 1-h exposures, which have been utilised most commonly, incubate spheroids in agar-coated (0.5–1%) Petri dishes or in glass universal containers. For longer exposure times, incubate the spheroids in a spinner vessel which will keep them in continuous suspension and prevent adherence to the vessel walls.

(iii) At the end of incubation rinse the spheroids in two to three 5-min washes of drug-free medium.

(i) *Volume growth delay.* Treated spheroids can be grown as a mass culture and the volume of individual randomly chosen spheroids determined at set time intervals. However, from a statistical view-point it is recommended that successive measurements are made on individually isolated spheroids placed in either 24-well or 96-well multidishes with agar-coated bases. The smaller wells can be used for spheroids up to 600 µm in diameter.

(1) Plate out 12–24 spheroids per drug concentration, and 24 controls into individual wells. For a 24-well plate use 0.5 ml of 1% agar for the base and add the spheroid in 1 ml of growth medium.

(2) At 2–3 day intervals measure two diameters (x,y) at right angles to each other, using an eye piece graticule in an inverted microscope. Calculate the size of the spheroid as 'mean diameter' ($\sqrt{x.y}$) or as 'volume'

$$\left[\frac{4}{3}\pi\left(\frac{\sqrt{x\cdot y}}{2}\right)^3\right].$$

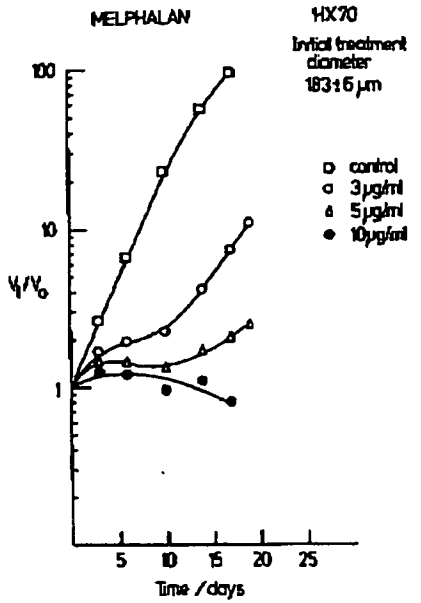

Figure 7. The effect of increasing concentrations of a 1-h exposure of melphalan on the growth of adenocarcinoma lung-derived spheroids. V_t = volume at time interval shown; V_o = volume at start of experiment. Significant differences ($p < 0.001$; student's 't' test) were observed at day 14 between control and 3.0 μg/ml, and 3.0 μg/ml and 10.0 μg/ml. The diameter shown on the figure is the mean diameter ± s.e. of the plated spheroids. (Reproduced with permission of the publishers, 54.)

and use to construct growth curves for drug-treated and control spheroids. Results are usually normalised to pre-treatment spheroid size and expressed as V_t/V_o where V_t = volume at time t and V_o = initial volume. An example is shown in *Figure 7* for spheroids derived from the xenograft of an adenocarcinoma of the lung. The method assumes that the spheroid is symmetrical, but flattening of spheroids to a dome-shape has been observed for some cell lines, which will lead to over-estimation of volume.

(ii) *Clonogenic growth.*

(1) Disaggregate spheroids to a single cell suspension using appropriate conventional treatment with 0.25% trypsin, or 0.125% trypsin plus 1000 U/ml type I CLS grade collagenase (Worthington).

(2) Adjust the cell number to 20–200/ml for cell lines, or up to 10^3/ml for primary cloning. If cloning in suspension use 10^4/ml for cell lines and up to 5×10^5/ml for primary cloning.

(3) Score for colonies when the designated size criterion in controls has been attained.

(iii) *Outgrowth as cell monolayer.* Obtaining a pure single cell suspension may be virtually impossible, and plating efficiencies may be very low. The following method circumvents these problems.

(1) Allow individual spheroids to attach to individual wells of tissue culture plastic microtitration plates.

(2) After 2–3 weeks remove the central spheroid and determine the number of cells in the monolayer which has proliferated from the spheroid. This may be done directly using cell counts of the detached cell population. Alternatively, the methodology described for quantitation of drug effect on cell monolayers in Sections 9.4.2 and 9.4.3 could be adopted.

9.4 Cytotoxicity Assays

9.4.1 *Protein Determination for Cell Monolayers*

The following protocol is based on the procedure described by Oyama and Eagle (30), for test-tube cultures.

Reagents

Solution A	20 g sodium carbonate 4 g sodium hydroxide 0.2 g sodium potassium tartrate	in 1 litre distilled water
Solution B	0.5 g copper sulphate 100 ml distilled water	

Store A and B at 4°C.

Solution C	50 ml A + 1 ml B (make immediately before use)
Solution D	1 part Folin-Ciocalteau phenol reagent plus 1 part distilled water.

Serial dilutions of bovine serum albumin, 10–100 μg/ml, for construction of standard curve.

(i) Wash the cell sheet three times in PBS.
(ii) Invert the tubes and drain for 20 min.
(iii) Add 5 ml of solution C to each tube and leave at room temperature for 20 min.
(iv) Add 1 ml of distilled water to each test and blank and 1 ml of albumin to standards.
(v) Jet in 0.5 ml of solution D using a hypodermic needle and syringe, to ensure initial rapid mixing.
(vi) Mix the tubes on a rotamixer.
(vii) Read absorption on colorimeter at 660 mμ after 2 h. Include a reagent blank containing 5 ml of solution C, 1 ml of distilled water and 0.5 ml of solution D; because some protein binding from growth medium to glass/plastic occurs, the reagent blank should be prepared in tubes which have contained growth medium but no cells. The standard curve loses linearity at high protein concentrations and a dilution factor may therefore be needed when cell density is high. Results should be converted to protein concentration using the standard curve, before expressing drug-treated tubes as a percentage of the control.

This method may be adapted to multi-well plates reading absorbance in microcuvettes in a conventional spectrophotometer, or to 96-well microtitration plates reading absorbance on one of various plate readers available (Flow, Gibco, Ilacon).

9.4.2 *Determination of Amino Acid Incorporation in Cell Monolayers, Growing in Microtitration Plates*

The following protocol describes a cytotoxicity assay of intermediate duration and is based on a procedure first outlined by Freshney *et al.* (29). Exposure and recovery conditions may be varied to suit requirements.

(i) Add 200 µl of cell suspension to each well of a 96-well microtitration plate. The cell density is dependent to some extent on the rate of proliferation of the cell type, and the length of the assay. It is desirable that confluence is not reached in control wells before the end of the assay, because their proliferation rate would then slow down relative to drug-treated wells, and confluent monolayers may detach from the well surface. For HeLa cells an initial concentration of 2×10^4/ml has been used, whilst for cell suspensions prepared from human ovarian tumour biopsies a higher concentration of $1-2 \times 10^5$/ml is recommended. Evaporation of medium from outer wells may occur, especially in longer assays, and it is therefore generally advised that the outer wells contain growth medium but no cells, in order to avoid the 'edge effect'. Edge effects due to evaporation can be reduced by using plate sealers (Mylar, Flow Laboratories). Some drugs are volatile, or give off volatile metabolites (e.g., formamides release formaldehyde), and this can cause variable non-specific cytotoxicity in adjacent wells. The use of plate sealers is essential for testing compounds such as these. Incubate the plates in a humidified atmosphere of air/5% CO_2.

(ii) When the cells have attached to form a dividing monolayer (24 h for cell lines, but full adherence may take longer for primary and secondary cultures) replace the medium and dilute the drugs serially across the plate, using a minimum of three wells per drug concentration. Use one row for controls containing growth medium only.

(iii) Incubate the plates with drugs for the chosen time period. If periods in excess of 24 h are to be used, add fresh drug solutions at 24-h intervals. Alternatively shorter exposures, e.g. 1 h, may be used, repeating daily if required.

(iv) At the end of incubation, remove the drugs and gently wash the cell monolayers twice in PBS; add fresh medium (200 µl) to each well.

(v) Incubate for the chosen recovery period; if this exceeds 3 days, change the medium at 3-day intervals, and check that the control wells have not attained confluence.

(vi) At the end of the recovery period add 50 µl of 5–20 µCi/ml [^{3}H]leucine (L-4,5 [^{3}H]leucine, Amersham plc) in growth medium to each well and incubate for 3 h.

(vii) Remove the isotope and wash the cell monolayers three times in PBS. Care must be taken to avoid damaging the monolayers during pipetting procedures; this is best done by tilting the plate to an angle of 45°, and inserting the pipette tip in the angle between the side and base of the well.

(viii) Add 100 µl of PBS and 100 µl of methanol to each well, remove and add 100 µl of pure methanol to each well. Fix the cells for 30 min, which prevents detachment of the cell monolayer during subsequent processing.

(ix) Remove the methanol and air-dry the plates. Fixed plates can be stored for at least 48 h at 4°C before further processing.

(x) Put the plates onto ice and wash the monolayers in three 5 min washes of ice-cold 10% trichloroacetic acid (TCA). Following fixation, wash solutions can be removed by inversion of the plate and shaking sharply once or twice.

(xi) Wash off the TCA with methanol, air-dry the monolayer and add 100 µl of 1 N NaOH to each well; leave overnight at room temperature for the protein to solubilise.

(xii) Transfer the 100 µl of NaOH to individual minivials placed in glass scintillation vials.

(xiii) Add 2.4 ml of scintillant (e.g., Fisofluor, Fisons Ltd.) to each vial followed by 100 µl of 1.1 N HCl to acidify the contents.

(xiv) Cap the vials, mix to homogenise and clarify the contents, and count for 5–10 min on a β-counter.

(xv) Express the results as a percentage of control c.p.m.

Some cell populations obtained from human tumour biopsy material are poorly adherent, although they will proliferate in suspension. The protocol described is still feasible, providing that the plates are centrifuged at 1000 r.p.m. for 15 min prior to medium removal to avoid cell loss.

9.4.3 *Measurement of Amino Acid Incorporation into Protein Using Autofluorography*

The previous methodology using liquid scintillation counting is labour-intensive during processing, and can cause problems when many plates are processed, particularly when β-counting facilities are restricted. Minivials tend to lose scintillant through their walls after some days storage and, although this can be reduced by storing at 4°C prior to counting, it does mean that samples should be counted as soon as possible after preparation. An alternative method, which is well suited to automation, has been developed by Freshney *et al.* (43).

(i) Perform the procedures described in Section 9.4.2 (i)–(x) using 5 µCi/ml of [^{35}S]methionine (42 Ci/mol) to label the cells in step (vi); [^{3}H]leucine can be used but longer development times are required.

(ii) Add 50 µl of toluene-based scintillant to each well and centrifuge the open plates in microtitration plate carriers at 800 r.p.m. for 1 h at room temperature. Even evaporation of scintillant is obtained using this method which gives a flat layer of scintillant on the base of the well, and therefore improves resolution of spots. Sodium salicylate may be substituted for toluene-based scintillant, avoiding solubilisation of the plate and evolution of potentially toxic toluene vapour during evaporation.

(iii) Place a sheet of X-ray film under the plate in a dark room, and secure with a layer of polyurethane sponge and a pressure plate (metal or glass) using adhesive tape or bulldog clips. Expose the plates in a light-proof box at −70°C with desiccant. An exposure time of 5 days gives an absorbance of 0.92 when 3000 cells per well are exposed to 10 µCi/ml of [^{35}S]methionine (84 µCi/mol).

(iv) Remove the film and develop for 5 min in Kodak D19 at 20°C. Fix in Ilfofix for 4 min, wash in Hypo clearing agent for 2 min, and tap water for 5 min. Dry the film.

(v) The results are quantitated by scanning the images on a scanning densitometer (e.g., Chromoscan, Joyce Loebl, Gateshead, UK) with a thin-layer attachment using an 11 mm circular aperture and a blue (465 nm) filter. ID_{50} values can be obtained directly from the O.D. readings.

9.4.4 *Incorporation of [^{3}H]Nucleotides into DNA/RNA*

The following procedure describes a short-term (3 h) cytotoxicity assay.

(i) Adjust the cell concentration to 10 times the final desired viable cell concentration, usually $10^5 - 5 \times 10^5$ per ml.

(ii) Titrate drug solutions at 900 µl per tube using replicates of three per concentration, including appropriate controls, and add 100 µl cell suspension per tube.

(iii) Incubate the tubes at 37°C for 2 h (see Section 8.2) and add isotopes (2.5 µCi/ml) at the end of the second hour [e.g., 6-[^{3}H]uridine (22 Ci/mmol); methyl-[^{3}H]-thymidine (5 Ci/mmol); 6-[^{3}H]deoxyuridine (25 Ci/mmol)]. Incubate for a further hour.

(iv) Centrifuge the tubes and wash the cells three times in PBS to remove drug and isotope. It is essential that an adequate wash procedure is included, in order to exclude non-specific activity.

Further processing depends on available facilities. The simplest and most reliable method involves filtration of cells onto glass fibre filters under vacuum, followed by extraction with TCA, washing and drying. A range of devices are available including a single filtration unit which fits on a Buchner flask (Millipore UK, Ltd.), a multiple filtration head (Millipore UK, Ltd.) and various devices made for automated harvesting of microtitration plates, which come with adaptors for test tubes (Flow Laboratories, Dynatek, Ilucon). The single unit is not suitable for multiple samples because of the time involved in processing each sample. The basic procedure is as follows.

(i) Harvest the cells onto glass-fibre filter paper, using 2 – 3 washes of the incubation vessel to ensure complete cell recovery.

(ii) Wash the filters three times with 5 ml of ice-cold 10% TCA.

(iii) Wash the filters sequentially with methanol, methanol-ether, and ether to remove water.

(iv) Dry the filters and transfer them to scintillation vials with 10 ml of an emulsifer cocktail scintillant [e.g., Beckman Ready Solv HP (High Performance)].

(v) Leave the samples for at least 1 h in the dark before counting to remove chemiluminescence, which produces artefactually high counts.

(vi) Count the samples for 5 – 10 min or as long as is required to reduce the counting error to ±5%.

Problems associated with the use of heterogeneous counting systems, as described above, have been detailed in an excellent technical review (55).

If filtration devices are not available, processing may be carried out in the incubation vessels, which should ideally be of glass.

i) Pellet the washed cells by centrifugation.

(ii) Add 1 ml of ice-cold 10% TCA, resuspend the cells and leave them to precipitate for 5 min on ice. Precipitate the pellet and repeat the TCA extraction twice.

(iii) Wash the pellet in methanol, methanol-ether, and ether to remove water and dry.

(iv) Add 1 ml of scintillation fluid to the residue which will dissolve, and transfer to scintillation vial. Make the volume up to 10 ml and count as before.

(i) *Modifications.* The following procedure has been described for measuring [^{3}H]-thymidine incorporation into DNA in cells growing in liquid suspension over soft agar (37).

(i) Add 25 µl of methyl-[^{3}H]thymidine (6.7 Ci/mM diluted to 75 µCi/ml in PBS) to cells for 24 h.

(ii) Harvest the cells and wash in PBS.

(iii) Add 5% TCA at 4°C to the washed cells for 30 min.

(iv) Centrifuge and repeat (step 3) for 10 min.

(v) Wash in methanol.

(vi) Resuspend the pellet in 0.5 ml of 10 × hyamine hydroxide, and heat at 60°C for 1 h.

(vii) Place in 12 ml of Hydrofluor scintillation fluid and count.

9.4.5 *Total DNA Synthesis Measured by [^{125}I]UdR Uptake*

The methodology is based on descriptions in the literature (39).

(i) Add 0.06 µCi/ml [^{125}I]UdR to growth medium of monolayer cultures for 24 h.

(ii) Wash the cultures three times in saline and add fresh growth medium.

(iii) After 6 h, repeat the wash procedure to remove unbound label and label released from lysed dead cells, and count bound ^{125}I activity in a gamma counter.

As for the microtitration assay, confluence should be avoided in control tubes because overcrowding leads to reduced [^{125}I]UdR incorporation, and therefore underestimation of cell kill. When cells have been grown as monolayers in inclined test tubes the upper limit of the assay was found to be about 2×10^5 cells, and the lower limit about 2×10^4 cells.

10. INTERPRETATION OF RESULTS

10.1 Relationship Between Cell Number and Cytotoxicity Index

The validity of a cytotoxicity index is dependent upon the degree of linearity between cell number and the chosen cytotoxicity parameter, and this should be established for any cytotoxicity assay. In clonogenic assays a linear relationship may not occur at low cell numbers due to the dependence of clonogenic growth on conditioning factors, whilst at high cell densities linearity is lost due to nutritional deficiencies. In cytotoxicity assays linearity may be lost at the upper end due to density-dependent inhibition of the relevant metabolic pathway, whilst the sensitivity limit of the assay may affect linearity at the lower end. This would cause apparent stimulation at low drug levels and an overestimation of cell kill at higher concentrations. Control cell numbers at the end of the assay must therefore fall on the linear portion of the curve. The accuracy at high levels of cell kill is dependent upon the range over which linearity extends, and influences

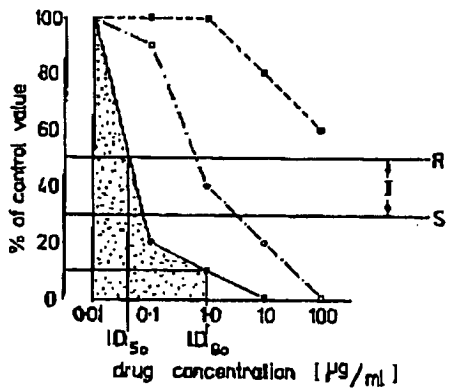

Figure 8. Interpretation of results using linear plot of response against drug concentration. ID_{50} and ID_{90} = concentration required to reduce cytotoxicity index to 50% or 90% of control value. ED = area under curve between 0 and 1 μg/ml (for peak plasma concentration of 10 μg/ml). S = cut-off boundary for sensitivity; R = cut-off boundary for resistance; I = intermediate zone. ●—●, sensitive population; ○—○, intermediate population; ■—■, resistant population.

the number of decades of cell kill which can be measured. If results are plotted on a log-scale this implies that the assay is accurate down to 3–4 decades of cell kill, which should be confirmed before expressing results in this way. This is particularly important in *in vitro* drug combination studies when synergism or additivity is often observed beyond the second decade of cell kill.

10.2 Dose-reponse Curves

Results are commonly plotted as dose-response curves using a linear scale for percentage inhibition (of isotope incorporation, for example) and a log scale for surviving fractions in clonogenic tests. Assay variation for replicate points is routinely depicted as mean ± standard deviation; a minimum of three replicates is therefore required for each test point. Some means is required for defining the sensitivity of a cell population in relation to other cell populations, or different test conditions: several parameters are available and are shown in *Figure 8*.

10.2.1 *Area Under Curve (AUC)*

The use of AUC recognises the probability that the shape of the dose-response curve may be instrumental in influencing the outcome to chemotherapy, rather than cell kill at any one concentration. It is calculated using the trapezoidal method which adds the area of rectangles and triangles under the survival curve. The method has been applied most extensively in the 'Human Tumour Stem Cell Assay' (56).

10.2.2 *Cut-off Points for Definition of Sensitivity and Resistance*

If plots of dose-response curves from multiple tumours show that tumours maintain their relative sensitivity rankings at different concentrations (i.e., crossing-over of dose-

response curves is minimal), then information on the relative sensitivities of different tumours can be obtained by defining sensitivity at one concentration, and this is the most commonly used method for *in vitro* predictive testing. When retrospective correlations between *in vitro* data are made for defining these cut-off points, an intermediate zone is found where tumours cannot be defined as sensitive or resistant, and there is no clear-cut correlation between *in vitro* results and clinical response. The size of the intermediate zone will be at least partly related to the inherent variability of the assay, larger zones being associated with higher standard deviations. Although sensitivity may be defined at one concentration it is recommended that more than one concentration is tested, particularly in the developmental stage of an assay.

10.2.3 *ID_{50} and ID_{90} Values*

Tumour sensitivity may also be defined by the ID_{50} and ID_{90} values (i.e., drug concentration required to inhibit viability by 50% or 90%).

10.2.4 *Correlation Between in Vitro and In Vivo Results*

Criteria for defining tumours as sensitive or resistant are based on retrospective correlations between *in vitro* results and clinical responses, using a 'training set' of data. Even when a laboratory is using an established method for tumour sensitivity testing, 'own laboratory' sets of training data should be obtained to allow for inter-laboratory variation. The response of patients with tumours of intermediate sensitivity may be influenced by prognostic factors other than tumour sensitivity (e.g., tumour burden at onset of chemotherapy, stage of disease, histology, tumour cell doubling time, previous chemotherapy and performance status). When analysing results for correlations some attempt to stratify patients according to these parameters may assist in providing more meaningful data. Quantitative assessment of tumour response is also of paramount importance. It is pointed out that *in vitro* chemosensitivity can be expected only to indicate that *some* degree of cell kill will be achieved *in vivo*, not that the patient will achieve a complete response to treatment, the latter being under the influence of other factors also. The true positive correlation rate of an assay is defined as

$$\frac{S/S + S/R}{S/S} \times 100\%$$

where numerator of each fraction = *in vitro* response and denominator = *in vivo* response. The true negative rate is defined as

$$\frac{R/R + R/S}{R/R} \times 100\%$$

In assessing the significance of the correlation rates obtained, these should be compared with the correlation rates which would be obtained were the *in vitro* results randomly distributed (57). For example, a drug gives a 50% response rate *in vivo*, and 50% of tumours show *in vitro* sensitivity to this drug. If the *in vitro* results are randomly distributed between sensitivity and resistance, then the chances of obtaining a positive correlation between *in vitro* sensitivity and *in vivo* response are 50% of 50% (i.e., 25%), and also of obtaining a positive correlation between *in vitro* resistance and *in vivo* resistance. The overall apparent positive correlation rate is therefore 50%. *In*

vitro versus in vivo correlations are also complicated by the use of combination regimes to treat patients. Strictly speaking, correlations should be made only when *in vitro* data is available for all drugs used. Whether or not they are tested in combination depends on the treatment protocol since some drugs are administered sequentially. Also if the assay can only measure two decades of cell kill it may be too insensitive to detect additive or synergistic effects.

11. PITFALLS AND TROUBLE SHOOTING

Problems which may be encountered with these assays include:

(i) large standard deviations;
(ii) variability between assays done on the same cell population;
(iii) stimulation to above control levels.

11.1 Large Standard Deviations

Possible reasons for large standard deviations include:

(i) faults in aliquotting cell suspension, which are most likely to be due to inadequate mixing of cell suspension during dispensing leading to uneven distribution of cells between replicates;
(ii) the presence of large cell aggregates in the original cell suspension, leading to uneven distribution of cells between replicates;
(iii) non-specificity of cytotoxicity end-point [e.g., due to measurement of non-specific binding of radioactivity (see Section 9.4.4)].

11.2 Inter-assay Variation

Replicate assays on different days cannot be performed on human tumour biopsy material to check day to day reproducibility, but this can be evaluated using cell lines. It is a recognised problem that cell lines which show consistent sensitivity profiles may show 'deviant' results occasionally, for reasons which cannot be identified. Specific reasons for failure to obtain reproducible results may include:

(i) failure to harvest the cell population at an identical time point (e.g., exponential growth *versus* early confluence *versus* late confluence);
(ii) deterioration of stock drug solutions (see Section 5);
(iii) when drug solutions have a short half-life they must be used immediately after diluting to ensure consistency in the drug levels available to cells in each assay;
(iv) failure to standardise incubation conditions (Section 9.2).

The assay system must be checked for reproducibility before applying it to human biopsy material.

11.3 Stimulation to Above Control Levels

Stimulation can be a true measure of cellular events but may be due to technical artefacts. These include:

(i) non-specific binding of radioactivity;
(ii) density-dependent inhibition of metabolic pathways in controls which is not evident in test situations where some cell kill has been achieved;

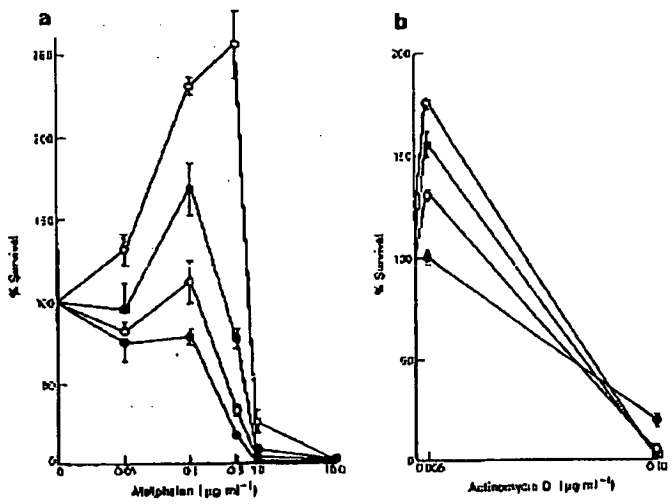

Figure 9. The effect of growth unit size on the survival curves of a murine melanoma cell line (CCL) to melphalan (a) and of a human melanoma biopsy to actinomycin D (b). Growth unit size and frequency was measured using FAS II automated image analysis system. ●, ≥60 μm; ○, ≥104 μm; ■, ≥124 μm; □, ≥149 μm. Mean ± s.e. shown. (Reproduced with permission of the publishers, 58.)

(iii) stimulation of uptake of metabolic precursors by anti-metabolite (e.g., thymidine by 5-fluorouracil and methotrexate).

Recent results (58) have shown that plated cell density influences the distribution in size of growth units in clonogenic assays, with large units decreasing as plated cell numbers increase. The effect of this on drug sensitivity profiles was examined and, as expected, the dose-response curve was strongly influenced by the size criterion used for colony-scoring, with stimulation to above control levels occurring when large colonies were scored (*Figure 9*).

11.4 Cytotoxicity versus Anti-neoplastic Activity

When *in vitro* tests are being used as a pre-screen for anti-neoplastic activity it is important that a distinction can be made between non-specific cytotoxicity and specific anti-neoplastic activity since the *in vitro* test gives no measure of the therapeutic index of the drug. Guidelines for distinguishing between the two are suggested in *Table 5*, although it is realised that exceptions exist.

12. AUTOMATION

Automation is a necessary technical development for large-scale screening of drugs and tumours. Of the assays described none except possibly the microtitration plate assay can be automated during the manipulative procedures involved in setting up the assay. Cytotoxicity assays involving the use of radioisotopes are the most readily quantitated

Table 5. Suggested Guidelines for Distinguishing Between Non-specific Cytotoxicity and Anti-neoplastic Activity.

Cytotoxicity	*Anti-neoplastic activity*
Equally effective against dividing and non-dividing cell populations	More effective against dividing cell populations than non-dividing cell populations
No heterogeneity of response between different tumour cell populations	Heterogeneity of response between different tumour cell populations
No difference in response of normal cells and tumour cells	Tumour cells more sensitive than normal cells
Primary biochemical lesions due to interference with cellular energy metabolism, or generation of free radicals	Primary biochemical lesions are: (i) inhibition of DNA replication or RNA transcription by binding to DNA; (ii) inhibition of nucleotide biosynthesis

automatically, though this may still require a high level of technical commitment which is alleviated by the use of the autofluorographic method described in Section 9.4.3. Various image analysers are also available for automated colony counting. Recently developed techniques for measuring the electrical impedance of cell cultures allows non-destructive determination of cytotoxicity at different time-points and is fully automated; such a technique has been used successfully to measure the drug sensitivity of a variety of cell populations (59).

13. FUTURE DEVELOPMENTS

In vitro cytotoxicity testing is a comparatively new concept in the field of safety evaluation and there are several aspects which merit investigation for evaluation of the role of these assays. These include:

(i) development of defined bioactivation systems for metabolic conversion to cytotoxic metabolites;
(ii) assay of agents with known cytotoxic activity for retrospective comparisons of *in vitro* effect;
(iii) development of culture systems for growth of normal target cells;
(iv) identification of the relevant *in vitro* cytotoxicity assay for compounds with differing modes of action, and intercomparison of different assay methods;
(v) identification of mechanisms of cytotoxicity.

The increasing optimism regarding the role of *in vitro* cytotoxicity testing in cancer chemotherapy is reflected in the ever-increasing amount of literature on the subject, which has risen dramatically with the recent development of clonogenic assay systems for human biopsy material. An international conference was held in 1983 which provided comprehensive coverage of the present situation in *in vitro* predictive testing (60). Evidence for the retrospective accuracy of prediction is well-established, but the ultimate proof of the value of these assays awaits the results of a random prospective trial in which the response rates of previously untreated patients treated on the basis of either clinical choice or laboratory results are compared. Because of the large number of patients required for such a study, a multicentre trial is an essential requisite. Other aspects

which need further development include:

(i) identification of specific growth factors to increase the incidence of successful *in vitro* growth;
(ii) establishment of more cell lines from different tumour types;
(iii) screening of new anti-neoplastic drugs and potentially active drugs (e.g., second generation agents), against cell lines of human tumour origin;
(iv) use of *in vitro* cytotoxicity testing for the design of drug combinations, evaluation of dose-dependency and optimum dose-scheduling;
(v) isolation of resistant lines from treated patients for studies on mechanisms of developed resistance; and
(vi) cloning of cell lines from untreated patients for identification of heterogeneous drug sensitivity and mechanisms of primary resistance.

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